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(54) Title: METHODS FOR SCREENING OSTEOGENIC COMPOUNDS TARGETING SYK KINASE AND/OR VAV3 AND USES OF SYK MODULATORS AND/OR VAV MODULATORS

(57) Abstract: Methods for screening osteogenic compounds, particularly compounds which result in net bone formation, including compounds which modulate osteoclast differentiation and function, using the Syk kinase pathway and/or the Vav3 pathway. The present invention relates particularly to methods for screening osteogenic compounds using the Dap12/Syk complex, Syk kinase or the Syk kinase pathway, the Vav pathway, Vav3, or the Vav3 pathway. The invention also provides methods and compositions for modulating the processes of bone formation and/or bone loss, thereby providing novel treatments for bone diseases. The present invention encompasses compounds identified by such screening methods and compositions comprising these compounds. The invention also provides methods for enhancing processes of bone formation, comprise administering compounds or agents which modulate, particularly inhibit, Syk kinase, Vav3 or the Syk kinase pathway or Vav3 pathway.



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**METHODS FOR SCREENING OSTEOGENIC COMPOUNDS TARGETING  
SYK KINASE AND/OR VAV3 AND USES OF SYK MODULATORS AND/OR  
VAV MODULATORS**

**GOVERNMENTAL SUPPORT**

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**FIELD OF THE INVENTION**

[0002] The present invention relates to methods for screening osteogenic compounds, including compounds which modulate osteoclast differentiation and function, using the Dap12 pathway and/or the Vav3 pathway. The present invention relates particularly to methods for screening osteogenic compounds using the Dap12/Syk complex, Syk kinase, the Syk kinase pathway, Vav, Vav3 or the Vav3 pathway. The present invention also relates to methods and compositions for modulating the processes of bone formation and/or bone loss, thereby providing novel treatments for bone diseases. The present invention further encompasses compounds identified by such screening methods and compositions comprising these compounds.

**BACKGROUND OF THE INVENTION**

[0003] Various conditions and diseases which manifest themselves in bone loss or thinning are a critical and growing health concern. It has been estimated that as many as 30 million Americans and 100 million worldwide are at risk for osteoporosis alone (Mundy et al. (1999) Science 286:1946-1949). Other conditions known to involve

bone loss include juvenile osteoporosis, osteogenesis imperfecta, hypercalcemia, hyperparathyroidism, osteomalacia, osteomalacia, osteolytic bone disease, osteonecrosis, Paget's disease of bone, bone loss due to rheumatoid arthritis, inflammatory arthritis, osteomyelitis, corticosteroid treatment, metastatic bone diseases, periodontal bone loss, bone loss due to cancer, age-related loss of bone mass, and other forms of osteopenia. Additionally, new bone formation is needed in many situations, e.g., to facilitate bone repair or replacement for bone fractures, bone defects, plastic surgery, dental and other implantations and in other such contexts.

[0004] Bone is a dense, specialized form of connective tissue. Bone matrix is formed by osteoblast cells located at or near the surface of existing bone matrix. Bone is resorbed (eroded) by another cell type known as the osteoclast (a type of macrophage). These cells secrete acids, which dissolve bone minerals, and hydrolases, which digest its organic components. The osteoclast originates by hematopoietic precursors of the monocyte/macrophage family migrating to the bone environment, where, in the presence of the cytokines RANKL and M-CSF, they multinucleate and assume the unique osteoclast phenotype thus acquiring the capacity to degrade mineralized matrix [Boyle et al., 2003; Teitelbaum, 2000]. Thus, bone formation and remodeling is a dynamic process involving an ongoing interplay between the creation and erosion activities of osteoblasts and osteoclasts (Alberts, et al., Molecular Biology of the Cell, Garland Publishing, N.Y. (3rd ed. 1994), pp. 1182-1186).

[0005] A key development in the field of bone cell biology is the recent discovery that RANK ligand (RANKL, also known as osteoprotegerin ligand (OPGL), TNF-related activation induced cytokine (TRANCE), and osteoclast differentiation factor (ODF)), expressed on stromal cells, osteoblasts, activated T-lymphocytes and mammary epithelium, is the unique molecule essential for differentiation of macrophages into osteoclasts (Lacey, et al. (1998) Cell 93: 165-176). The cell surface receptor for RANKL is RANK, Receptor Activator of Necrosis Factor (NF)-kappa B. RANKL is a type-2 transmembrane protein with an intracellular domain of less than about 50 amino acids, a transmembrane domain of about 21 amino acids, and an

extracellular domain of about 240 to 250 amino acids. RANKL exists naturally in transmembrane and soluble forms. The deduced amino acid sequence for at least the murine, rat and human forms of RANKL and variants thereof are known. See e.g., Anderson, et al., U.S. Pat. No. 6,017,729, Boyle, U.S. Pat. No. 5,843,678, and Xu J. et al., J. Bone Min. Res. (2000) 15:2178) which are incorporated herein by reference. RANKL (OPGL) has been identified as a potent inducer of bone resorption and as a positive regulator of osteoclast development. Lacey et al., supra.

**[0006]** RANK signaling, activated by its ligand RANKL which is expressed on stromal cells and osteoblasts [Suda et al., 1999], is mediated by a series of protein kinases including c-Src, c-Jun N terminal kinase (JNK), p38, extracellular signal related kinase (ERK), phosphoinositol-3-kinase (PI-3K), and those activating NF-kB [Darnay et al., 1998; Galibert et al., 1998; Lee et al., 2002; Matsumoto et al., 2000]. M-CSF, which via its receptor, c-Fms, simulates many of the same pathways, promotes proliferation of osteoclast precursors and survival of the mature resorptive cell [Tanaka et al., 1993; Woo et al., 2002]. Together, therefore, RANKL and M-CSF induce expression of genes, such as those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor and  $\beta 3$  integrin, which characterize the mature osteoclast and its committed precursors [Faccio et al., 2003b; Kudo et al., 2002]

**[0007]** The fact that osteoclasts are derived from macrophages, cells which are fundamental to immune recognition, has led to a series of experiments which link the immune system to osteoclast recruitment and function. For example, T-lymphocyte-produced cytokines, including RANKL and  $\text{TNF}\alpha$ , appear central to the enhanced osteoclastogenesis responsible for the bone loss attending menopause and the peri-articular bone erosions of rheumatoid arthritis [Cenci et al., 2000; Romas et al., 2002; Weitzmann et al., 2000]. In this context, the process of antigen presentation, itself, is also a fundamental event in pathological osteoclastogenesis [Jenkins et al., 2002].

[0008] These and other insights gained into the means by which osteoclast precursors differentiate and how the mature polykaryon resorbs bone have led to the identification of a number of new anti-osteoporosis therapeutic targets including cathepsin K, c-Src and the  $\alpha v \beta 3$  integrin, thus encouraging the exploration of other candidates [Wilder, 2002; Zaidi et al., 2003]. Among the most promising of such potential targets are intraosteoclastic signaling molecules which also function in the immune system [Kaifu et al., 2003; Paloneva et al., 2002].

[0009] Dap12 is a transmembrane adapter molecule expressed in a variety of cells of the immune system [Lanier and Bakker, 2000; Tomasello et al., 1998]. In myeloid cells, Dap12 pairs with surface residing receptors including TREMs [Colonna, 2003]. The cytoplasmic domain of Dap12 contains the immunoreceptor tyrosine-based activation ITAM motif, which functions as a docking site for tyrosine kinases, including Syk [McVicar et al., 1998]. Interestingly, deletion of the Dap12 gene, in man, results in Nasu-Hakola disease, which includes skeletal abnormalities in its phenotype [Kaifu et al., 2003; Kondo et al., 2002]. Furthermore, Dap12 is expressed by osteoclasts and, as evidenced by the development of osteopetrosis in mice lacking the protein, is essential for normal osteoclast function [Kaifu et al., 2003; Kondo et al., 2002].

[0010] Spleen tyrosine kinase (Syk) is a non-receptor type of protein tyrosine kinase expressed by cells of the immune system. The Syk family of tyrosine kinases include Syk and Zap-70, which are structurally distinct from other kinases in containing a C-terminal kinase domain and tandem N-terminal SH2 domains that bind phosphorylated immunoreceptor tyrosine-based activation motifs (ITAMs). Both Syk and Zap-70 are critical for immune function. When the tyrosines on Syk are phosphorylated, they act as docking sites for proteins such as phospholipase  $C\gamma 1$  (PLC $\gamma$ ), VAV and CBL, which may be substrates for Syk (Turner M. et al (2000) Immunol Today 21(3):148-154). Syk is expressed by all hematopoietic cells and is essential for lymphocyte development and signal transduction via immune receptors in non-lymphoid cells. Gene targeting studies have demonstrated an essential role for

Syk in signaling through a variety of immune receptors in both lymphoid and myeloid cells (including Ig $\alpha$ , TCR $\zeta$ , CD3 $\epsilon$ , Fc $\epsilon$ RI $\beta$ , Fc $\epsilon$ RI $\gamma$ , thrombin, von Willebrand factor, GPVI collagen receptor and integrin  $\alpha_{\text{IIb}}\beta_3$ ) (Turner M et al (1995) *Nature* 378:298-302; Turner M. et al (2000) *Immunol Today* 21(3):148-154; Colucci F et al (2002) *Nature Immun* 3:288-294; Mocsai, A et al (2002) *Immunity* 16:547-558). Although similar in overall structure, there are certain differences between Syk and Zap-70. In particular, the activation of Zap-70 by immune receptors requires Src-family kinases, whereas Syk can function in a Src-family kinase-independent manner (Chu DH et al (1998) *Immunol Rev* 165:167-180).

[0011] Vav proteins are GDP/GTP exchange factors (GEFs) for Rho/Rac GTPases that are activated by tyrosine phosphorylation, catalyzing the exchange of nucleotides on these GTP binding proteins and thereby facilitating their transition from the inactive (GDP-bound) to the active (GTP-bound) state (Bustello X.R. (2000) *Mol Cell Biol* 20:1461-1477; for review see Turner M and Billadeau DD (2002) *Nature Reviews* 2:476-486). Rho and Rac GTPases participate in coordinated cellular responses to extracellular stimuli and are important in promoting formation of cytoskeletal structures, the activation of serine/threonine kinase cascades and in induction of gene expression. The enzymatic activity of Vav proteins is tightly regulated by direct tyrosine phosphorylation, with Vav proteins remaining inactive in the absence of stimuli and becoming rapidly activated and transiently phosphorylated upon receptor activation. Several tyrosine kinases, including Lck, Fyn, Hck and Syk, have been shown to phosphorylate the Vav family of GEFs (Deckert M et al (1996) *Immunity* 5:591-604; Han J et al (1997) *Mol Cell Biol* 17:1346-1353; Schuebel KE et al (1998) *EMBO J* 17:66-8-6621; Michel F et al (1998) *J Biol Chem* 273:31932-31938; Huang J et al (2000) *Proc Natl Acad Sci USA* 97:10923-10929). Several lines of evidence indicate that the Vav proteins are crucial for developmental, mitogenic and pathological processes. Impaired development, lymphopenia and defective immune responses of B and T lymphocytes is observed on elimination of the Vav gene in mice (Fischer, KD et al (1995) *Nature* 374:474-477; Tarakhovsky A. et al (1995) *Nature* 374:467-470; Turner M et al (1997) *Immunity* 7:451-460; Zhang R. et

al (1995) Nature 374:470-473). The phenotypes of Vav1<sup>-/-</sup> versus Vav2<sup>-/-</sup> and the double knockout Vav1<sup>-/-</sup> Vav2<sup>-/-</sup> demonstrate that the Vav proteins are required for the peripheral maturation and/or survival of B cells and that Vav2 deficiency does not affect T cell development, while Vav1 is required for proper peripheral T cell function and signaling. Thus, in the immune system Vav1 is very important and Vav2 and Vav3 cannot compensate functionally for the loss of Vav1 (Turner M and Billadeau DD (2002) Nature Reviews 2:476-486).

**[0012]** The Vav family is distributed phylogenetically in all animals, with known members in nematodes (*C. elegans*) flies (*D. melanogaster*) and mammals (Vav, Vav2 and Vav3) (Bustello X.R. (2000) Mol Cell Biol 20:1461-1477). The Vav3 gene, encoding a 98kDa Vav3 protein, was most recently identified and cloned from a human placental cDNA library and the protein demonstrates 69.4 and 66.1% overall sequence similarity with the human Vav and Vav2 proteins, respectively (Movilla N and Bustello XR (1999) Mol Cell Biol 19(11):7870-7885). Although sharing the same substrates and containing an identical domain structure, including a DBL-homology domain, a Calponin-homology domain, a zinc-finger domain, a SRC-homology 2 (SH2) domain, and two SRC-homology 3 (SH3) domains, the Vav proteins differ in their affinities towards specific Rho/Rac proteins. Vav 3 works substoichiometrically on RhoG and RhoA and at higher concentrations on Rac-1, while Vav works only at higher concentrations on RhoA and in substoichiometric amounts on Rac1 and RhoG (Movilla N et al (2001) Oncogene 20:8057-8065).

**[0013]** Vav1 is expressed primarily in cells of the hematopoietic system. Vav2 displays a ubiquitous pattern of expression during embryonic and adult mouse stages (Schuebel KE et al (1996) Oncogene 13:363-371). Vav3 is similarly widely expressed in human tissues, including spleen, brain, peripheral blood lymphocytes, thymus, heart, kidney, liver, placenta, lung and in embryonic hematopoietic tissues (Movilla N and Bustello XR (1999) Mol Cell Biol 19(11):7870-7885). Transient expression of truncated Vav3 proteins induces a rapid reorganization of the actin cytoskeleton, leading to membrane ruffling, lamellipodia, and the formation of thin bundles of

stress fibers. However, unlike Vav2, Vav3 does not show transforming activity on expression in NIH 3T3 cells although morphological change is observed (Movilla N and Bustello XR (1999) Mol Cell Biol 19(11):7870-7885). A potential role of Vav3 in the regulation of cell division has been suggested and Fujikawa et al have demonstrated that Vav3 expression is regulated in a cell cycle-dependent manner and that deregulated expression of Vav3, but not Vav1 or Vav2 in HeLa cells disrupts cell division and leads to multinucleated cells (Fujikawa K et al (2002) Proc Natl Acad Sci USA 99(7):4313-4318).

[0014] Accordingly, a need exists for new methods of screening osteogenic compounds, compounds which enhance net bone formation, including compounds which modulate osteoclast differentiation and function. In particular there is a need for novel targets in osteoclast differentiation and function. Further, there is a need for novel compositions that are useful in modulating osteoclast differentiation and function, thereby modulating the process of bone loss and net bone formation.

[0015] The citation of references herein shall not be construed as an admission that such is prior art to the present invention.

### SUMMARY OF THE INVENTION

[0016] The invention relates to the application and use of modulators, including inhibitors, of Syk kinase and/or Vav to modulate osteoclast differentiation and function. The invention particularly relates to the application and use of modulators, including inhibitors, of Vav3 to modulate osteoclast differentiation and function. In an additional aspect, the invention relates to the application and use of modulators, including inhibitors, of guanine exchange factors (GEFs) to modulate osteoclast differentiation and function. The invention relates to the use of modulators, particularly inhibitors, of Syk kinase and/or of Vav3 for amelioration or treatment of bone disease, particularly wherein it is desired to reduce or control osteoclast function and differentiation, including but not limited to osteoporosis, juvenile osteoporosis,

osteogenesis imperfecta, hypercalcemia, hyperparathyroidism, osteomalacia, osteomalacia, osteolytic bone disease, osteonecrosis, Paget's disease of bone, bone loss due to rheumatoid arthritis, inflammatory arthritis, osteomyelitis, corticosteroid treatment, metastatic bone diseases, periodontal bone loss, bone loss due to cancer, age-related loss of bone mass, other forms of osteopenia, as well as in instances where facilitation of bone repair or replacement is desired such as bone fractures, bone defects, plastic surgery, dental and other implantations.

**[0017]** In a particular aspect, the invention provides methods of modulating the differentiation and/or function of osteoclasts by administration of a compound or agent that blocks or otherwise inhibits the Syk kinase pathway. In one aspect, a method for modulating the differentiation and/or function of osteoclasts is provided whereby an effective amount of an inhibitor of Syk kinase is administered.

In a particular aspect, the invention provides methods of modulating the differentiation and/or function of osteoclasts by administration of a compound or agent that blocks or otherwise inhibits the Vav pathway, particularly the Vav3 pathway. In one aspect, a method for modulating the differentiation and/or function of osteoclasts is provided whereby an effective amount of an inhibitor of Vav3 is administered.

In a still further aspect, the invention provides methods of modulating the differentiation and/or function of osteoclasts by administration of one or more compound or agent that blocks or otherwise inhibits one or more of or both of the Vav pathway, particularly the Vav3 pathway, and the Syk kinase pathway. In one aspect, a method for modulating the differentiation and/or function of osteoclasts is provided whereby an effective amount of one or more inhibitor of Vav3 and/or Syk kinase is administered.

**[0018]** In a further aspect, the invention provides a method for treating bone disease in a mammal comprising administering to said mammal an effective amount one or

more of a Syk kinase inhibitor and a Vav3 inhibitor. In a particular aspect, the invention further provides a method for treating osteoporosis in a mammal comprising administering to said mammal an effective amount of one or more of a Syk kinase inhibitor and a Vav3 inhibitor. In a still further aspect a method is provided for reducing the risk of non-traumatic bone fracture in a mammal comprising administering to said mammal an effective amount of one or more of a Syk kinase inhibitor and a Vav3 inhibitor.

**[0019]** In a further aspect, the invention provides a method for treating bone disease in a mammal comprising administering to said mammal an effective amount of a Syk kinase inhibitor in combination with one or more other compounds for the treatment of bone disease. In particular, an effective amount of a Syk inhibitor may be used in combination with one or more anti-resorptive or anabolic compound for treatment of bone disease, including but not limited to a bisphosphonate, a Vav3 or Vav3 pathway inhibitor, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, a supplemental bone formation agent parathyroid hormone (PTH) or its derivative or fragments thereof, PTH related protein (PTHrp), a bone morphogenetic protein, osteogenin, NaF, PGE<sub>2</sub> agonists, a statin, and a RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomerized form of RANKL .

In a further aspect, the invention provides a method for treating bone disease in a mammal comprising administering to said mammal an effective amount of a Vav3 inhibitor in combination with one or more other compounds for the treatment of bone disease. In particular, an effective amount of a Vav3 inhibitor may be used in combination with one or more anti-resorptive or anabolic compound for treatment of bone disease, including but not limited to a bisphosphonate, a Syk or Syk pathway inhibitor, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, a supplemental bone formation agent parathyroid hormone (PTH) or its derivative or fragments thereof, PTH related protein (PTHrp), a bone morphogenetic protein, osteogenin, NaF, PGE<sub>2</sub> agonists, a statin, and a RANK

ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomerized form of RANKL .

In an additional aspect, the invention provides a method for treating bone disease in a mammal comprising administering to said mammal an effective amount of one or more of a Syk kinase and a Vav3 inhibitor in combination with each other, and additionally with or without one or more other compounds for the treatment of bone disease. In particular, an effective amount of a Vav3 inhibitor and/or a Syk kinase inhibitor may be used in combination with one or more anti-resorptive or anabolic compound for treatment of bone disease, including but not limited to a bisphosphonate, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, a supplemental bone formation agent parathyroid hormone (PTH) or its derivative or fragments thereof, PTH related protein (PTHrp), a bone morphogenetic protein, osteogenin, NaF, PGE<sub>2</sub> agonists, a statin, and a RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomerized form of RANKL .

[0020] The object of the invention extends to the provision of methods for screening for osteogenic compounds, particularly compounds which modulate the differentiation or function of osteoclasts, by using some portion of the Dap12 pathway or the Vav 3 pathway. In a particular object, methods are provided for screening compounds which modulate osteoclast differentiation and/or function, by modulating the Dap12/Syk complex, Syk kinase, the Syk kinase pathway, by modulating GEFs, Vav, Vav3, or the Vav3 pathway. In addition, the present invention encompasses compounds that are identified by the screening methods disclosed herein. Further provided are methods and compositions for modulating osteoclast differentiation and function and thereby modulating bone loss and net bone formation.

[0021] A method of the invention involves screening osteogenic compounds and includes the steps of selecting compounds that modulate some portion of the Dap12/Syk kinase pathway or the Vav3 pathway and performing osteoclast assays with said compounds. The method of screening of the present invention may also

include the step of identifying compounds that led to reduction of osteoclast numbers or osteoclast activity, or alternatively, to an increase in bone formation in the presence of osteoclasts and osteoblasts in said assays. A compound which inhibits the Dap12/Syk kinase pathway, including an inhibitor of Syk kinase, or the Vav pathway, including an inhibitor of Vav3, is suitable for use in therapy and modulation of bone disease, including osteoporosis or other diseases or conditions where bone loss is enhanced or altered. Conversely, a compound which activates the Dap12/Syk kinase pathway, including an activator or agonist of Syk kinase, or activates the Vav3 pathway, including an activator or agonist of Vav3, may be suitable in therapy or modulation of bone disease, including bone cancer and bone metastases or altered bone formation, where it is desired to activate osteoclasts, bone destruction or enhance bone loss.

**[0022]** Selecting compounds that modulate Syk kinase, the Syk kinase pathway, Vav3, the Vav3 pathway, may involve different assays, such as, e.g., phosphorylation assays wherein a compound's ability to block phosphorylation of or by Syk or Vav or enhance dephosphorylation of Syk or a Syk target or a Vav3 or Vav3 target is determined directly or wherein the activity of a molecule downstream of Syk or of Vav3 or which is modulated or activated by Syk or upon Syk phosphorylation (including but not limited to phospholipase C $\gamma$ 1 (PLC $\gamma$ ), VAV, CBL, ERK and JNK) or modulated or activated by Vav3 or upon Vav3 phosphorylation (including but not limited to Rho and Rac).

**[0023]** Compounds for screening may be selected from various libraries of small molecular weight compounds, peptides, or alternatively may be selected by homology modeling, computational modeling, and screening phage display libraries.

**[0024]** Another method of the present invention involves screening osteogenic compounds and include the steps of incubating the desired compounds with osteoclasts, osteoclast precursors, or related cell lines and determining the differentiation, maturation activation and functional status of said cells. The

compounds to be screened include compounds selected for their ability to bind to or otherwise interact with Dap12 or Syk kinase or Vav3 as well as those which activate and/or inactivate at least some portion of the Syk kinase pathway regardless of Syk kinase stimulation or of the Vav3 pathway regardless of Vav3 stimulation. Activation of osteoclasts or osteoclast precursors can be determined by establishing the activation status of intracellular proteins in the incubated cells, whose activation is indicative of osteoclast differentiation and/or function. The assays used to test for activation of intracellular proteins vary according to the protein in question and are well established in the art, e.g. Western blots, kinase and phosphatase assays. When activation is observed, i.e. a protein in its activated state, it may designate the compound being tested as having the net ability to activate and/or induce proliferation of osteoclasts, osteoclast precursors or related cell lines. If activation of intracellular proteins is not seen in the assay, i.e. a protein not in its activated state or in a deactivated state, the compound being tested is determined as having the net ability to inactivate and/or block or reduce proliferation, differentiation or function of osteoclasts, osteoclast precursors or related cell lines.

[0025] In one preferred embodiment, the modulation of intracellular proteins on the Dap12/Syk kinase pathway or the Vav/Vav3 pathway constitutes phosphorylation of the same. Specifically, the phosphorylated proteins include Syk kinase and/or Vav3 and its target proteins, including kinases, which are phosphorylated or otherwise interact with or are modulated by Syk kinase and/or Vav3. In one embodiment the proteins include, but are not limited to phospholipase C $\gamma$ 1 (PLC $\gamma$ ), VAV, CBL, ERK and JNK which may be substrates for Syk or activated or more active in the presence of phosphorylated Syk, and Rho and Rac, which may be substrates for Vav3 or activated or more active in the presence of phosphorylated Vav3.

[0026] The invention further provides methods of screening osteogenic compounds based on their ability to modulate the tyrosine phosphorylation of Syk kinase, including but not limited to by modulating a or any kinase which is capable of phosphorylating tyrosine(s) on Syk or Zap-70, or on Vav3 as determined from in vitro

assays. Preferably, the kinases are Syk kinase specific, specific for the Syk kinase family including Syk and Zap-70, or are specific for a protein downstream from and activated or phosphorylated by Syk or via the Syk kinase pathway. Preferably, the kinases are Vav3 specific, specific for the Vav family including Vav1, Vav2 and Vav3, or are specific for a protein downstream from and activated or phosphorylated by Vav3 or via the Vav3 pathway.

[0027] In addition, the invention provides methods of screening osteogenic compounds based on their ability to modulate phosphatase(s) as determined from in vitro assays. In a particular aspect, the phosphatases are Syk kinase specific, specific for the Syk kinase family including Syk and ZAP70, or are specific for a kinase downstream from and activated or phosphorylated by Syk or via the Syk kinase pathway. In a particular aspect, the phosphatases are Vav3 specific, specific for the Vav family including Vav1, Vav2 and Vav3, or are specific for a kinase downstream from and activated or phosphorylated by Vav3 or via the Vav3 pathway.

[0028] The present invention also includes compositions for modulating bone formation, particularly for modulating the differentiation and/or function of osteoclasts. These compositions may comprise compounds identified by the screening methods disclosed herein. Thus, these compositions may be used to treat diseases or conditions characterized at least in part by the loss of bone mass.

[0029] Various phosphorylation or kinase inhibitors, including specific Syk or Syk kinase family inhibitors, and GEF or Vav family inhibitors, have been identified and are known in the art. Examples of Syk inhibitors in the art include but are not limited to: organic purine derivative inhibitors described by Collingwood et al. (U.S. Patent 6,589,950); 2-pyrimidineamine derivatives of Davis et al. (U.S. Patent 6,352,029); 1,6 Naphthyridines inhibitors disclosed in WIPO Publication No. WO 03/057695A1; the orally available Syk inhibitor 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride (denoted BAY 61-3606) (Yamamoto, N. et al (2003) J. Pharmacol Exp Ther 306(3): 1174-1181); piceatannol

(3, 4, 3', 5'-tetradroxy-trans-stilbene), a Syk-selective tyrosine kinase inhibitor (Seow, CJ et al (2002) Eur J Pharmacol 443(1-3):189-196); thiazole compounds are described in WO 02/096905A1; heterocyclic carboxamide derivatives disclosed in EP 1 184 376 A1; and antisense oligonucleotide to Syk kinase (Stenton, G.R. et al (2002) J Immunol 169(2):1028-1036). Particularly preferred are compounds or agents which inhibit GEFs, most particularly inhibitors that inhibit the Vav family (Vav1, Vav2 and Vav3) with some specificity, still more particularly inhibitors which have specificity or preference for Vav3. For instance, Gewirtz describes antisense oligonucleotides to Vav and their ability to inhibit the proliferation of malignant but not normal cells (U.S. Patent 5,612,212, which is incorporated herein by reference in its entirety). EP1223216, which is incorporated herein by reference in its entirety, describes intracellular nucleic acid inhibitors of small guanine-nucleotide-exchange-factors. The invention provided herein includes the use of these inhibitors for the modulation and/or treatment of bone disease, including for instance, but not limited to, osteoporosis, juvenile osteoporosis, osteogenesis imperfecta, hypercalcemia, hyperparathyroidism, osteomalacia, osteomalacia, osteolytic bone disease, osteonecrosis, Paget's disease of bone, bone loss due to rheumatoid arthritis, inflammatory arthritis, osteomyelitis, corticosteroid treatment, metastatic bone diseases, periodontal bone loss, bone loss due to cancer, age-related loss of bone mass, other forms of osteopenia, as well as in instances where facilitation of bone repair or replacement is desired such as bone fractures, bone defects, plastic surgery, dental and other implantations.

**[0030]** The invention includes an assay system for screening of potential drugs effective to modulate Syk activity and/or Vav3 activity of target mammalian cells by interrupting or potentiating the Syk kinase, Syk kinase pathway, Vav3 or Vav3 pathway. In one instance, the test drug could be administered to a cellular sample to determine its effect upon the kinase activity or phosphorylation status of Syk or of Vav, particularly Vav3, by comparison with a control.

[0031] The assay system could more importantly be adapted to identify drugs or other entities that are capable of binding to Syk kinase or Vav3, either in the cytoplasm or in the nucleus, thereby inhibiting or potentiating Syk kinase or Vav3 activity.

[0032] The present invention includes an assay system which may be prepared in the form of a test kit for the quantitative analysis of the extent of Syk kinase or Zap-70 activity or amount of phosphorylated Syk or Zap-70, or to identify drugs or other agents that may mimic or block Syk or Zap-70 activity or phosphorylation. The system or test kit may comprise a labeled component prepared by one of the radioactive and/or enzymatic techniques discussed herein or known to the skilled artisan, coupling a label to the Syk or Zap-70 kinase, their agonists and/or antagonists, and one or more additional immunochemical reagents, at least one of which is a free or immobilized ligand, capable either of binding with the labeled component, its binding partner, one of the components to be determined or their binding partner(s).

The present invention includes an assay system which may be prepared in the form of a test kit for the quantitative analysis of the extent of Vav3 activity or amount of phosphorylated Vav3, or to identify drugs or other agents that may mimic or block Vav3 activity or phosphorylation. The system or test kit may comprise a labeled component prepared by one of the radioactive and/or enzymatic techniques discussed herein or known to the skilled artisan, coupling a label to the Vav3, their agonists and/or antagonists, and one or more additional immunochemical reagents, at least one of which is a free or immobilized ligand, capable either of binding with the labeled component, its binding partner, one of the components to be determined or their binding partner(s).

[0033] It is a still further object of the present invention to provide pharmaceutical compositions for use in therapeutic methods for modulation of bone disease which comprise one or more of a Syk kinase inhibitor and a Vav3 inhibitor. In a further aspect, the invention provides pharmaceutical compositions for use in therapeutic methods for modulation of bone disease which comprise a Syk kinase inhibitor and

one or more anti-resorptive or anabolic compound for treatment of bone disease, including but not limited to a bisphosphonate, a Vav3 or Vav3 pathway inhibitor, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, a supplemental bone formation agent parathyroid hormone (PTH) or its derivative or fragments thereof, PTH related protein (PTHrp), a bone morphogenetic protein, osteogenin, NaF, PGE<sub>2</sub> agonists, a statin, and a RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomerized form of RANKL. In a further aspect, the invention provides pharmaceutical compositions for use in therapeutic methods for modulation of bone disease which comprise a Vav3 inhibitor and one or more anti-resorptive or anabolic compound for treatment of bone disease, including but not limited to a bisphosphonate, a Syk or Syk pathway inhibitor, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, a supplemental bone formation agent parathyroid hormone (PTH) or its derivative or fragments thereof, PTH related protein (PTHrp), a bone morphogenetic protein, osteogenin, NaF, PGE<sub>2</sub> agonists, a statin, and a RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomerized form of RANKL. In a still further aspect, the invention provides pharmaceutical compositions for use in therapeutic methods for modulation of bone disease which comprise one or more of a Vav3 inhibitor and a Syk kinase inhibitor and one or more anti-resorptive or anabolic compound for treatment of bone disease, including but not limited to a bisphosphonate, a Syk or Syk pathway inhibitor, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, a supplemental bone formation agent parathyroid hormone (PTH) or its derivative or fragments thereof, PTH related protein (PTHrp), a bone morphogenetic protein, osteogenin, NaF, PGE<sub>2</sub> agonists, a statin, and a RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomerized form of RANKL.

[0034] Other objects and advantages will become apparent to those skilled in the art from a review of the following description which proceeds with reference to the following illustrative drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0035] **FIGURE 1** is an image of a Western blot depicting the rapid activation of the members of the MAPK pathway in murine osteoclast precursors following the treatment of cells with GST-RANKL. The activation was measured at the time of GST-RANKL/RANK interaction (0 minutes) and 5, 15, and 30 minutes following the interaction. From the top, the second, fourth, and sixth panels show the total levels of JNK, p38, and ERK respectively. The first, third, and fifth panels depict the phosphorylated (activated) forms of JNK, p38, and ERK respectively.

[0036] **FIGURE 2** is an image of a Western blot depicting the activation of Akt in murine osteoclast precursors following the treatment of cells with GST-RANKL. The activation was monitored at the time of GST-RANKL/RANK interaction, and 5 and 15 minutes following the interaction. The bottom panel depicts the levels of total Akt at specified time points, whereas the top panel depicts the phosphorylated forms of Akt.

[0037] **FIGURE 3** depicts that M-CSF partially rescues osteoclastogenesis of Dap12<sup>-/-</sup> cells. Wild type (WT) and Dap12<sup>-/-</sup> osteoclasts were generated from BMMs cultured for 5 day with RANKL (100ng/ml) and low (10ng/ml) or high (100ng/ml) dose M-CSF. Images represent TRAP stained cultures of osteoclasts at 100X or 200X magnification. Within 5 days WT cells form numerous large multinucleated cells independent of M-CSF concentration. Although less pronounced, many Dap12<sup>-/-</sup> cells become large, multinucleated and TRAP positive only when cultured in the presence of high dose M-CSF.

[0038] **FIGURE 4** depicts defective proliferation of Dap12<sup>-/-</sup> cells. WT and Dap12<sup>-/-</sup> BMMs or pre-osteoclasts were cultured for 3 days in the presence of increasing concentrations of M-CSF after which proliferation was evaluated by BrdU incorporation or MTT assay. The proliferative response of Dap12<sup>-/-</sup> cells is diminished particularly as the concentration of cytokine increases.

[0039] **FIGURE 5A and 5B** depicts that Dap12<sup>-/-</sup> osteoclasts fail to organize their cytoskeleton or resorb mineralized matrix. WT and Dap12<sup>-/-</sup> osteoclasts were generated on dentine slices in the presence of RANKL and high dose M-CSF. After five days, the cells were fixed and stained with FITC-phalloidin to visualize actin organization (**A**) or removed to identify bone resorption pits (**B**). While WT osteoclasts form numerous well defined actin rings (**A**, arrows) and resorptive pits (**B**, arrows), Dap12<sup>-/-</sup> osteoclasts exhibit a disorganized cytoskeleton (**A**) and fail to resorb bone (**B**).

[0040] **FIGURE 6** shows that high dose M-CSF induces expression of osteoclast specific mRNAs by Dap12<sup>-/-</sup> cells. WT and Dap12<sup>-/-</sup> BMMs were cultured for 4 days with RANKL and 10ng/ml (L) or 100ng/ml (H) M-CSF. The indicated osteoclastogenic markers were analyzed by PCR. Untreated BMMs (day 0) do not express osteoclast markers. After two days in low or high M-CSF, WT cells express cathepsin K, MMP-9 and calcitonin receptor. At each day, expression of these markers by Dap12<sup>-/-</sup> cells is diminished in low M-CSF but normalized in high M-CSF.

[0041] **FIGURE 7A, 7B and 7C** depict that RANKL and M-CSF signaling is normal in Dap12<sup>-/-</sup> pre-osteoclasts. BMMs stimulated with RANKL (**A**) and pre-osteoclasts exposed to low (**B**) or high dose M-CSF (**C**) for up to one hour, were subjected to immunoblot to detect activation of the indicated proteins. Phosphorylation of p-38, AKT and I $\kappa$ B $\alpha$  in response to RANKL, and I $\kappa$ B $\alpha$  degradation and re-synthesis occurs equally in WT and Dap12<sup>-/-</sup> BMMs. Phosphorylation of ERK and activation of c-Fos

in pre-osteoclasts stimulated with low or high dose M-CSF is similar in WT or Dap12<sup>-/-</sup> pre-osteoclasts.  $\beta$ -actin serves as loading control.

**[0042] FIGURE 8A and 8B** depict spreading on and migration to osteopontin (OPN) by Dap12<sup>-/-</sup> pre-osteoclasts is abnormal. (A) WT and Dap12<sup>-/-</sup> preOCs were plated on OPN coated coverslips. While WT cells adhere and spread onto OPN within 30 min, Dap12<sup>-/-</sup> pre-osteoclasts attach to the protein but do not spread (insert). Adding M-CSF increases adhesion of both cell types and rescues the spreading defect of Dap12<sup>-/-</sup> cells. (B) Pre-osteoclast migration was assessed using transwells in which the lower membrane was coated with OPN, in the absence or presence of high dose M-CSF as chemoattractant. While M-CSF increases the directed migration of both cell types, the motility of Dap12<sup>-/-</sup> cells remains less than WT.

**[0043] FIGURE 9A and 9B** demonstrate that Syk regulates intracellular signaling and osteoclast formation. (A) WT and Dap12<sup>-/-</sup> pre-osteoclasts were maintained in suspension (S) or plated on OPN (O) for 30 min and Syk phosphorylation was analyzed by immunoblot. Cell adhesion increases Syk phosphorylation in WT but not in Dap12<sup>-/-</sup> cells. (B) Syk<sup>+/-</sup> and Syk<sup>-/-</sup> BMMs were maintained in the presence of RANKL and low or high dose M-CSF. Syk<sup>+/-</sup> osteoclasts form within 5 days in both conditions. In contrast, cells which are predominantly mononucleated and fail to spread normally, independent of M-CSF concentrations, form TRAP-expressing cells with few nuclei. Thus, osteoclastogenesis is arrested in the absence of Syk.

**[0044] FIGURE 10A and 10B** depicts that Syk<sup>-/-</sup> pre-osteoclasts adhere poorly and fail to fully activate Src and Pyk2. (A) Syk<sup>+/-</sup> and Syk<sup>-/-</sup> preOCs were plated on OPN coated dishes. While Syk<sup>+/-</sup> cells spread onto OPN within 30min, Syk<sup>-/-</sup> pre-osteoclasts do not spread on the protein. (B) Syk<sup>+/-</sup> and Syk<sup>-/-</sup> pre-osteoclasts were maintained in suspension (S) or plated on OPN (O) for 30 min and Src and Pyk2 phosphorylation was analyzed by immunoblot. Cell adhesion increases Src and Pyk2 phosphorylation in Syk<sup>+/-</sup> but not in Syk<sup>-/-</sup> cells.  $\beta$ -actin serves as loading control.

[0045] **FIGURE 11** depicts the amino acid sequence of human Syk.

[0046] **FIGURE 12** depicts the nucleic acid sequence of human Syk.

[0047] **FIGURE 13A and 13B** depict RNase protection assays to detect levels of the different Vav RNAs in osteoclasts and T cells. Expression of Vav1, Vav2 and Vav3 in osteoclast precursors (BMMs treated with M-CSF and RANKL for from 0 to 5 days) is depicted in **A**. In **A** the Vav1 expression is shown in a 10 fold longer exposure than for the Vav2 and Vav3 expression. Expression of Vav1, Vav2 and Vav3 in T cells is shown in **B**.

[0048] **FIGURE 14A, 14B and 14C** depicts radiographic and histological assessment of Vav knockout mice. **A** shows radiographic analysis of WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> mouse long bones. Histologic analysis of tibias fixed in formalin, decalcified for 4 days and processed is shown in **B**. Osteoclasts were stained for TRAP. **C** depicts histomorphometric analysis of trabecular bone volume versus total bone volume (BV/TV) for WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup>.

[0049] **FIGURE 15** depicts osteoclastogenesis as depicted by TRAP and actin staining. Bone marrow macrophages of WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> mice were treated with 100 ng/ml GST-RANKL and 10 ng/ml M-CSF. After 5 days, cells were fixed and stained for either TRAP or actin which reveals cytoskeletal reorganization.

[0050] **FIGURE 16A, 16B and 16C**. **A** provides immunofluorescence of WT, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts plated on dentin, and the actin ring visualized with phalloidin-FITC. **B** depicts quantitative analysis of osteoclast height measured from the basolateral membrane through the ruffled border. **C** presents the same osteoclasts of **A**, isolated from the dentin, with the bone resorptive pits stained with hematoxylin red.

**[0051] FIGURE 17A and 17B** depicts NF $\kappa$ B signaling in WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts in the presence of 100 ng/ml RANKL for 0, 5, 15 and 30 minutes as assessed by Western blot. In **A**, levels of I $\kappa$ B $\gamma$ , which is degraded upon NF $\kappa$ B signaling and resynthesized by 30 minutes, and  $\beta$ -actin are depicted. **B** depicts nuclear translocation of p65 in a Western blot of nuclear extracts from WT and Vav1.3<sup>-/-</sup> osteoclasts.

**[0052] FIGURE 18A and 18B** depicts M-CSF signaling in WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts in the presence of 100 ng/ml M-CSF for 0, 5 and 15 minutes as assessed by Western blot (**A**) and 0, 30 and 60 minutes as assessed by PCR (**B**). Pre-osteoclasts were generated from BMMs by incubation with RANKL and M-CSF for 2 days, and were then stimulated with M-CSF as noted. **A** provides a Western blot of p-ERK using a phospho-specific ERK antibody, with  $\beta$ -actin as a control. **B** shows levels of c-Fos, Fra-1 and GAPDH RNA in M-CSF treated osteoclasts as determined by PCR with c-Fos, Fra-1 and GAPDH specific primers.

**[0053] FIGURE 19** shows osteoclast (OC) marker expression in pre-osteoclasts from WT(1), Vav1<sup>-/-</sup>(2), Vav3<sup>-/-</sup>(3) and Vav1.3<sup>-/-</sup>(4). Total RNA was isolated from BMMs, cultured with RANKL and M-CSF for 2 days or for 4 days. mRNA levels for CathK, TRAP, CalcR and GAPDH RNA were determined by PCR using specific primers.

**[0054] FIGURE 20** depicts the morphology of TRAP stained osteoclasts from WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> mice after transduction with a control GFP or a GFP-Vav3 vector. Bone marrow macrophages of WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> were transduced with GFP or GFP-Vav3 vector. GFP positive cells were selected by FACS and incubated with RANKL and M-CSF to stimulate osteoclastogenesis. Osteoclasts were then TRAP stained.

**[0055] FIGURE 21** depicts the role of Syk in regulating tyrosine phosphorylation of Vav3. Pre-osteoclasts, generated by culturing BMMs from Syk<sup>+/-</sup> and Syk<sup>-/-</sup> marrow with M-CSF and RANKL, were lifted and either kept in suspension

(S) or replated on osteopontin (OPN) for 30 minutes. Lysates were prepared and immunoprecipitation was performed with a phospho-tyrosine specific 4-G10 antibody. Western blotting utilized a Vav3 monoclonal antibody (described herein) and a rabbit polyclonal Syk antibody.

**[0056] FIGURE 22A, 22B and 22C.** A is a Western blot of pre-osteoclasts (day2 in culture with RANKL and M-CSF) grown in suspension (S) or plated onto osteopontin (OPN) for 30 minutes (A). Western analysis was performed with p-ERK, p-Src or  $\beta$ -actin antibodies. B depicts a Western blot of M-CSF treated WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts to demonstrate the activated form of Rho. C depicts a Western blot of M-CSF treated WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts to characterise the activated form of Rac. In B and C, a pull down technique was utilized wherein protein A-Glutathione beads bound to an effector for activated Rho (RBD) or an effector for activated Rac (Pac-1) are used (Faccio et al 2003a) to isolate GTP-Rho and GTP-Rac, the proteins released from the beads by boiling, and then visualized by Western with Rho and Rac specific antibodies.

**[0057] FIGURE 23** depicts the amino acid sequence of human Vav3.

**[0058] FIGURE 24** depicts the nucleic acid sequence of human Vav3.

**[0059] FIGURE 25A and 25B** Adhesion of pre-osteoclasts via avb3 results in recruitment of both Syk and c-Src to the integrin. A. Purified BMMs were incubated with optimal M-CF and RANKL, lifted and either maintained in suspension (sus) or allowed to adhere to vitronectin (Adh). Cell lysates were immuno-precipitated with a polyclonal anti beta 3 antibody and immune complexes were separated by SDS-PAGE, transferred to PVDF and blotted with specific anti-sera to Syk or c-Src. Equal levels of the beta 3 integrin subunit act as loading controls. B. Cell lysates from suspended or adherent pre-osteoclasts were immuno-precipitated with anti-Syk and then subjected to western analysis as described in A, probing for c-Src. Equal levels of Syk act as loading controls. In each, the TCL lane shows total cell lysate proteins.

**[0060] FIGURE 26A and 26B** Re-introduction of avb3 into beta 3 null mice rescues binding of Syk and c-Src in pre-osteoclasts. Purified BMMs from beta 3 null mice were retrovirally transduced with a cDNA coding for full length beta 3 protein. Following selection with puromycin for three days, pre-osteoclasts were generated from surviving cells with M-CSF and RANKL. Cell lysates were immuno-precipitated with anti-Syk, followed by western blot for c-Src, as shown in **A**. Equal levels of beta actin act as loading controls. Total cell lysate (TCL) proteins are shown in **B**.

**[0061] FIGURE 27** Phosphorylation of Syk in pre-osteoclasts requires activation of avb3. Pre-osteoclasts were generated from BMMs with optimal M-CSF and RANKL and maintained in suspension (Sus) or allowed to adhere to vitronectin (Adh). Cell lysates were prepared and total cellular phospho-proteins were isolated by immuno-precipitation with excess Syk-specific antibody and equal amounts of protein in the two immune complexes were subjected to western blot analysis with antibody 4G10, a phospho-specific antibody. The TCL lane shows total cell lysate proteins.

**[0062] FIGURE 28** A complex containing Syk, c-Src and vav3 is formed following ligation of avb3 in pre-osteoclasts. Pre-osteoclasts were generated from BMMs with optimal M-CSF and RANKL and maintained in suspension (Sus) or allowed to adhere to vitronectin (Adh). Cell lysates were prepared and immuno-precipitated with excess Syk antibody. Western blot analysis was performed using antibodies specific for Syk, c-Src, and Vav3. Equal levels of Syk act as loading controls.

#### DETAILED DESCRIPTION

**[0063]** In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill

of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994)]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames & S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames & S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Freshney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

**[0064]** The terms "spleen tyrosine kinase", "Syk", "Syk kinase" and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to a non-receptor type of protein tyrosine kinase. As such, Syk as used herein includes and encompasses members of the Syk kinase family, particularly Syk and Zap-70. Syk refers to the proteinaceous material, including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in FIGURE 11 (SEQ ID NO:1), and the profile of activities set forth herein. Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "spleen tyrosine kinase", "Syk" and "Syk kinase(s)" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

**[0065]** "Syk pathway" and "Syk kinase pathway" refers to Syk and/or Zap-70 and the downstream proteins capable of being modulated, including activated, by a signal from Syk and/or Zap-70 or which are more active on a signal from Syk and/or Zap-70 or in the presence of phosphorylated Syk and/or Zap-70. Syk pathway and Syk kinase pathway further includes the upstream proteins, including particularly a

kinase(s), which are capable of phosphorylating tyrosine(s) on Syk and/or Zap-70 or involved in the signal which results in phosphorylated Syk and/or Zap-70. Exemplary downstream proteins include but are not limited to phospholipase C $\gamma$ 1 (PLC $\gamma$ ), VAV, CBL, ERK and JNK.

[0066] The terms "Vav" and "Vav3" and any variants not specifically listed, may be used herein interchangeably, and as used throughout the present application and claims refer to a guanine-nucleotide-exchange-factor. In particular, Vav or Vav3 is expressed in bone, particularly in osteoclasts and osteoclast precursors. Vav3 refers to the proteinaceous material, including single or multiple proteins, and extends to those proteins having the amino acid sequence data described herein and presented in FIGURE 13 (SEQ ID NO:1), and the profile of activities set forth herein.

Accordingly, proteins displaying substantially equivalent or altered activity are likewise contemplated. These modifications may be deliberate, for example, such as modifications obtained through site-directed mutagenesis, or may be accidental, such as those obtained through mutations in hosts that are producers of the complex or its named subunits. Also, the terms "Vav" and "Vav3" are intended to include within their scope proteins specifically recited herein as well as all substantially homologous analogs and allelic variations.

[0067] "Vav pathway" and "Vav3 pathway" refers to Vav3 and the downstream proteins capable of being modulated, including activated, by a signal from Vav3 and/or from its family members Vav1 and/or Vav2 or which are more active on a signal from Vav3 or in the presence of phosphorylated Vav3. Vav pathway and Vav3 pathway further includes the upstream proteins, including particularly a kinase(s), which are capable of phosphorylating tyrosine(s) on Vav3 and/or Vav1 or Vav2 or involved in the signal which results in phosphorylated Vav3 (for example an upstream signaling protein such as Syk). Exemplary downstream proteins include but are not limited Rho and Rac.

[0068] "Osteoclast precursor" refers to a cell or cell structure, such as a pre-osteoclast, which is any cellular entity on the pathway of differentiation between a macrophage and a differentiated and functional osteoclast. The term osteoclast includes any osteoclast-like cell or cell structure which has differentiated fully or partially from a macrophage, and which has osteoclast character, including but not limited to positive staining for tartrate-resistant acid phosphatase (TRAP), but which is not a fully differentiated or functional osteoclast, including particularly aberrantly differentiated or non functional osteoclasts or pre-osteoclasts.

[0069] "Osteoclast culture" refers to any *in vitro* or *ex vivo* culture or system for the growth, differentiation and/or functional assessment of osteoclasts or osteoclast precursors, whether in the absence or presence of other cells or cell types, for instance, but not limited to, osteoblasts, macrophages, hematopoietic or stromal cells.

[0070] "RANKL" or "RANK ligand" are used interchangeably herein to indicate a ligand for RANK (Receptor Activator of NF $\kappa$ B).

[0071] "RANK pathway" refers to RANK and the downstream proteins relating to bone formation capable of being activated by a signal from RANK and including NF $\kappa$ B translocation, proto-oncogene proteins, including c-Fos, and intracellular kinases, including ERK1/2, IKK, PI3 kinase, Akt, JNK, and p38.

[0072] "MAP kinase" or "MAPK" are used interchangeably herein, and are abbreviations for mitogen activated protein kinase. The MAPK family comprises three proteins, ERK1/2, JNK, and p38.

[0073] "ERK1/2" refers to ERK1 and ERK2, which are abbreviations for extracellular signal-regulated kinase 1 and extracellular signal-regulated kinase 2, respectively.

[0074] "JNK" is an abbreviation for c-jun N-terminal kinase.

[0075] "p38" is a kinase of 38 kDa, which is a member of the MAPK family of kinases.

[0076] "Akt" is Akt serine threonine kinase.

[0077] The terms "compound" and "molecule" are used interchangeably herein.

[0078] As used herein, "ERK1/2-specific phosphatase" refers to phosphatase(s), whose function is to dephosphorylate (inactivate) the active forms of ERK1/2.

[0079] As used herein, "NFκB phosphatase(s)" refers to phosphatase(s), whose function is to dephosphorylate (inactivate) the active forms of NFκB.

[0080] "JNK-specific phosphatase" refers to phosphatase(s), whose function is to dephosphorylate (inactivate) the active forms of JNK.

[0081] "p38-specific phosphatase" refers to phosphatase(s), whose function is to dephosphorylate (inactivate) the active forms of p38.

[0082] "Akt-specific phosphatase" refers to phosphatase(s), whose function is to dephosphorylate (inactivate) the active forms of Akt.

[0083] The phrase "effective amount" or "therapeutically effective amount" is used herein to mean an amount of the substance in question that one skilled in the art would expect a statistically significant effect. For example, an "effective amount" for therapeutic uses is the amount of the composition comprising an active compound herein required to provide a clinically significant increase in healing rates in fracture repair; reversal or inhibition of bone loss in osteoporosis; prevention or delay of onset of osteoporosis; stimulation and/or augmentation of bone formation in fracture non-unions and distraction osteogenesis; increase and/or acceleration of bone growth into prosthetic devices; repair or prevention of dental defects; or treatment or inhibition of

other bone loss conditions, diseases or defects, including but not limited to those discussed herein above. Such effective amounts will be determined using routine optimization techniques and are dependent on the particular condition to be treated, the condition of the subject, the route of administration, the formulation, and the judgment of the practitioner and other factors evident to those skilled in the art. The dosage required for the compounds of the invention (for example, in osteoporosis where an increase in bone formation and/or reduction in bone loss is desired) is manifested as that which induces a statistically significant difference in bone mass or bone loss between treatment and control groups. This difference in bone mass or bone loss may be seen, for example, as at least 1-2%, or any clinically significant increase in bone mass or reduction in bone loss in the treatment group. Other measurements of clinically significant increases in healing may include, for example, an assay for the N-terminal propeptide of Type I collagen, tests for breaking strength and tension, breaking strength and torsion, 4-point bending, increased connectivity in bone biopsies and other biomechanical tests well known to those skilled in the art. General guidance for treatment regimens may be obtained from experiments carried out in vitro or in animal models of the disease of interest.

[0084] "Treatment" refers to therapy, prevention and prophylaxis and particularly refers to the administration of medicine or the performance of medical procedures with respect to a subject or patient, for either prophylaxis (prevention) or to cure or reduce the extent of or likelihood of occurrence or the infirmity or malady or condition or event in the instance where the patient is afflicted. Thus, in treating a subject, the compounds of the invention may be administered to a subject already suffering from loss of bone mass or other bone disease as provided herein or to prevent or inhibit the occurrence of such condition.

[0085] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similar untoward reaction, such as gastric upset, dizziness and the like, when administered to a human.

[0086] As used herein, "pg" means picogram, "ng" means nanogram, "ug" or "μg" mean microgram, "mg" means milligram, "ul" or "μl" mean microliter, "ml" means milliliter, "l" means liter.

[0087] The amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is retained by the polypeptide. NH<sub>2</sub> refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide.

[0088] It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues.

[0089] A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

[0090] A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

[0091] A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g.,

restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

[0092] An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

[0093] A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

[0094] Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

[0095] A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible

for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes.

[0096] An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

[0097] A "signal sequence" can be included before the coding sequence. This sequence encodes a signal peptide, N-terminal to the polypeptide, that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

[0098] The term "oligonucleotide" is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

[0099] The term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use of the method. For example, for diagnostic applications, depending on the

complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

**[0100]** The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to hybridize therewith and thereby form the template for the synthesis of the extension product.

**[0101]** As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

**[0102]** A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

[0103] Two DNA sequences are "substantially homologous" when at least about 75% (preferably at least about 80%, and most preferably at least about 90 or 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified by comparing the sequences using standard software available in sequence data banks, or in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., *supra*; DNA Cloning, Vols. I & II, *supra*; Nucleic Acid Hybridization, *supra*.

[0104] It should be appreciated that within the scope of the present invention and useful in and for the methods of the present invention are DNA sequences which encode a Syk kinase, including for instance human Syk and Zap-70, or which encode a Vav3, including for instance human Vav3, particularly having the same amino acid sequence as that of FIGURE 11, SEQ ID NO:1, or FIGURE 23, SEQ ID NO:3 but which are degenerate to SEQ ID NO:1 or SEQ ID NO:3. By "degenerate to" is meant that a different three-letter codon is used to specify a particular amino acid. It is well known in the art that certain codons can be used interchangeably to code for each specific amino acid.

[0105] Mutations can be made in sequences of use in the methods and invention herein, including in Syk kinase (e.g., SEQ ID NO:1), or in Vav3 (e.g., SEQ ID NO:3) such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the

structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein.

[0106] The following is one example of various groupings of amino acids:

Amino acids with nonpolar R groups

Alanine, Valine, Leucine, Isoleucine, Proline, Phenylalanine, Tryptophan, Methionine

[0107] Amino acids with uncharged polar R groups

Glycine, Serine, Threonine, Cysteine, Tyrosine, Asparagine, Glutamine

[0108] Amino acids with charged polar R groups (negatively charged at Ph 6.0)

Aspartic acid, Glutamic acid

[0109] Basic amino acids (positively charged at pH 6.0)

Lysine, Arginine, Histidine (at pH 6.0)

[0110] Another grouping may be those amino acids with phenyl groups:

Phenylalanine, Tryptophan, Tyrosine

[0111] Another grouping may be according to molecular weight (i.e., size of R groups).

[0112] Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH<sub>2</sub> can be maintained.

[0113] Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced a potential site for disulfide bridges with another Cys. A His may be introduced as a

particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces -turns in the protein's structure.

[0114] Two amino acid sequences are "substantially homologous" when at least about 70% of the amino acid residues (preferably at least about 80%, and most preferably at least about 90 or 95%) are identical, or represent conservative substitutions.

[0115] A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

[0116] An "antibody" is any immunoglobulin, including antibodies and fragments thereof, that binds a specific epitope. The term encompasses polyclonal, monoclonal, and chimeric antibodies, the last mentioned described in further detail in U.S. Patent Nos. 4,816,397 and 4,816,567.

[0117] An "antibody combining site" is that structural portion of an antibody molecule comprised of heavy and light chain variable and hypervariable regions that specifically binds antigen.

[0118] The phrase "antibody molecule" in its various grammatical forms as used herein contemplates both an intact immunoglobulin molecule and an immunologically active portion of an immunoglobulin molecule.

[0119] Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contains the paratope, including those portions known in the art as Fab, Fab', F(ab')<sub>2</sub> and F(v), which portions are preferred for use in the therapeutic methods described herein.

[0120] Fab and F(ab')<sub>2</sub> portions of antibody molecules are prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See for example, U.S. Patent No. 4,342,566 to Theofilopolous et al. Fab' antibody molecule portions are also well-known and are produced from F(ab')<sub>2</sub> portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide. An antibody containing intact antibody molecules is preferred herein.

[0121] The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen; e.g., a bispecific (chimeric) monoclonal antibody.

[0122] A DNA sequence is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that DNA sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the DNA sequence to be expressed and maintaining the correct reading frame to permit expression of the DNA sequence under the control of the expression control sequence and production of the desired product encoded by the DNA sequence. If a gene that one desires to insert into a

recombinant DNA molecule does not contain an appropriate start signal, such a start signal can be inserted in front of the gene.

[0123] The term "standard hybridization conditions" refers to salt and temperature conditions substantially equivalent to 5 x SSC and 65°C for both hybridization and wash. However, one skilled in the art will appreciate that such "standard hybridization conditions" are dependent on particular conditions including the concentration of sodium and magnesium in the buffer, nucleotide sequence length and concentration, percent mismatch, percent formamide, and the like. Also important in the determination of "standard hybridization conditions" is whether the two sequences hybridizing are RNA-RNA, DNA-DNA or RNA-DNA. Such standard hybridization conditions are easily determined by one skilled in the art according to well known formulae, wherein hybridization is typically 10-20°C below the predicted or determined  $T_m$  with washes of higher stringency, if desired.

[0124] As is well known in the art, DNA sequences may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate unicellular host. Such operative linking of a DNA sequence of this invention to an expression control sequence, of course, includes, if not already part of the DNA sequence, the provision of an initiation codon, ATG, in the correct reading frame upstream of the DNA sequence. A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of use in this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences. Suitable vectors include derivatives of SV40 and known bacterial plasmids, e.g., *E. coli* plasmids col El, pCR1, pBR322, pMB9 and their derivatives, plasmids such as RP4; phage DNAs, e.g., the numerous derivatives of phage  $\lambda$ , e.g., NM989, and other phage DNA, e.g., M13 and filamentous single stranded phage DNA; yeast plasmids such as the 2 $\mu$  plasmid or derivatives thereof; vectors useful in eukaryotic cells, such as vectors useful in insect or mammalian cells; vectors derived from combinations of plasmids and phage DNAs, such as plasmids

that have been modified to employ phage DNA or other expression control sequences; and the like.

[0125] Any of a wide variety of expression control sequences -- sequences that control the expression of a DNA sequence operatively linked to it -- may be used in these vectors to express the DNA sequences of this invention. Such useful expression control sequences include, for example, the early or late promoters of SV40, CMV, vaccinia, polyoma or adenovirus, the *lac* system, the *trp* system, the *TAC* system, the *TRC* system, the *LTR* system, the major operator and promoter regions of phage  $\phi$ , the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase (e.g., Pho5), the promoters of the yeast -mating factors, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0126] A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi such as yeasts, and animal cells, such as CHO, R1.1, B-W and L-M cells, African Green Monkey kidney cells (e.g., COS 1, COS 7, BSC1, BSC40, and BMT10), insect cells (e.g., Sf9), and human cells and plant cells in tissue culture.

[0127] It will be understood that not all vectors, expression control sequences and hosts will function equally well to express the DNA sequences of use in this invention. Neither will all hosts function equally well with the same expression system. However, one skilled in the art will be able to select the proper vectors, expression control sequences, and hosts without undue experimentation to accomplish the desired expression without departing from the scope of this invention. In selecting an expression control sequence, a variety of factors will normally be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence or gene to be expressed,

particularly as regards potential secondary structures. Suitable unicellular hosts will be selected by consideration of, e.g., their compatibility with the chosen vector, their secretion characteristics, their ability to fold proteins correctly, and their fermentation requirements, as well as the toxicity to the host of the product encoded by the DNA sequences to be expressed, and the ease of purification of the expression products. Considering these and other factors a person skilled in the art will be able to construct a variety of vector/expression control sequence/host combinations that will express the DNA sequences of use in this invention.

[0128] It is further intended that Syk kinase analogs or Vav3 analogs may be prepared from nucleotide sequences of the protein complex/subunit derived within the scope of the present invention. Analogs, such as fragments, may be produced, for example, by pepsin or other suitable protease digestion of Syk or Vav3 material. Other analogs, such as muteins, can be produced by standard site-directed mutagenesis of Syk or Vav3 coding sequences. Analogs exhibiting "Syk activity" or "Vav3 activity" such as small molecules, whether functioning as promoters or inhibitors, may be identified by known *in vivo*, *ex vivo* and/or *in vitro* assays.

[0129] A DNA sequence encoding Syk or Vav3 can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the Syk or Vav3 amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge, *Nature*, 292:756 (1981); Nambair et al., *Science*, 223:1299 (1984); Jay et al., *J. Biol. Chem.*, 259:6311 (1984). Synthetic DNA sequences allow convenient construction of genes which will express Syk analogs or "muteins". Alternatively, DNA encoding muteins can be made by site-directed mutagenesis of native Syk genes or cDNAs, and muteins can be made directly using conventional polypeptide synthesis. A general method for site-specific incorporation of unnatural amino acids into proteins is described in Christopher J. Noren, Spencer J. Anthony-Cahill, Michael C. Griffith, Peter G. Schultz, *Science*,

244:182-188 (April 1989). This method may be used to create analogs with unnatural amino acids.

[0130] The present invention is based on applicants' discovery that Syk kinase and Vav3 each play a key and critical role in the differentiation and/or function of osteoclasts. In addition, their roles are interconnected, with the data herein demonstrating a functional linkage between integrin  $\alpha_v\beta_3$ -dependent adhesion and activation of a Syk/c-src/Vav3 signalling pathway that is central to osteoclast biology. In accordance with the present invention, applicants have discovered that Syk participates in osteoclast differentiation and cytoskeletal organization. In osteoclasts, Syk is required for differentiation and cell spreading. Thus, the invention relates to the application and use of modulators of Syk kinase to modulate osteoclast differentiation and function. In accordance with the present invention, applicants have discovered that Vav3 participates in osteoclast differentiation, osteoclast signalling and cytoskeletal organization. In osteoclasts, Vav3 is required for proper differentiation and function, and is responsible for correct bone architecture and bone homeostasis by acting downstream of the M-CSF receptor and of  $\alpha_v\beta_3$  integrin. Thus, the invention relates to the application and use of modulators of Vav3 to modulate osteoclast differentiation and function. By modulating osteoclast differentiation and/or function, it is possible to thereby modulate the extent and degree of bone loss, particularly in bone disease, such as osteoporosis, wherein it is desired to reduce the extent or amount of bone loss versus bone mass increase. In addition, it may be desired to modulate osteoclast differentiation and/or function to increase bone loss or to stimulate the destruction of bone in certain instances or in certain bone diseases such as in bone defects, in bone cancer or metastasis or in instances where it is desired to reduce or slow bone formation or inappropriate bone formation.

[0131] In particular, the invention relates to the use of inhibitors of Syk kinase or Vav3 to inhibit or block osteoclast differentiation and function, thereby inhibiting or reducing bone loss. The invention relates to the use of modulators, particularly inhibitors, of Syk kinase and/or of Vav3 for amelioration or treatment of bone disease,

particularly wherein it is desired to reduce or control osteoclast function and differentiation, including but not limited to osteoporosis, juvenile osteoporosis, osteogenesis imperfecta, hypercalcemia, hyperparathyroidism, osteomalacia, osteomalacia, osteomalacia, osteomalacia, osteomalacia, Paget's disease of bone, bone loss due to rheumatoid arthritis, inflammatory arthritis, osteomyelitis, corticosteroid treatment, metastatic bone diseases, periodontal bone loss, bone loss due to cancer, age-related loss of bone mass, other forms of osteopenia, as well as in instances where facilitation of bone repair or replacement is desired such as bone fractures, bone defects, plastic surgery, dental and other implantations.

[0132] In a particular aspect, the invention provides methods of modulating the differentiation and/or function of osteoclasts by administration of a compound or agent that blocks or otherwise inhibits the Syk kinase pathway and/or the vav3 pathway. In one aspect, a method for modulating the differentiation and/or function of osteoclasts is provided whereby an effective amount of one or more of an inhibitor of Syk kinase or Vav3 is administered.

#### Selecting Compounds or Agents

[0133] Various kinase inhibitors, including specific Syk or Syk kinase family inhibitors and Vav or Vav3 inhibitors, have been identified and are known in the art. Examples of Syk inhibitors in the art include but are not limited to: organic purine derivative inhibitors described by Collingwood et al. (U.S. Patent 6,589,950); 2-pyrimidineamine derivatives of Davis et al. (U.S. Patent 6,352,029); 1, 6 Naphthyridines inhibitors disclosed in WIPO Publication No. WO 03/057695A1; the orally available Syk inhibitor 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride (denoted BAY 61-3606) (Yamamoto, N. et al (2003) J. Pharmacol Exp Ther 306(3): 1174-1181); piceatannol (3, 4, 3', 5'-tetradroxy-trans-stilbene), a Syk-selective tyrosine kinase inhibitor (Seow, CJ et al (2002) Eur J Pharmacol 443(1-3):189-196); thiazole compounds are described in WO 02/096905A1; heterocyclic carboxamide derivatives disclosed in EP 1 184 376 A1;

and antisense oligonucleotide to Syk kinase (Stenton, G.R. et al (2002) J Immunol 169(2):1028-1036). Particularly preferred are compounds or agents which inhibit GEFs, most particularly inhibitors that inhibit the Vav family (Vav1, Vav2 and Vav3) with some specificity, still more particularly inhibitors which have specificity or preference for Vav3. For instance, Gewirtz describes antisense oligonucleotides to Vav and their ability to inhibit the proliferation of malignant but not normal cells (U.S. Patent No. 5,612,212, which is incorporated herein by reference in its entirety). EP1223216, which is incorporated herein by reference in its entirety, describes intracellular nucleic acid inhibitors of small guanine-nucleotide-exchange- factors. The invention provided herein includes the use of these inhibitors for the modulation and/or treatment of bone disease, including for instance osteoporosis, juvenile osteoporosis, osteogenesis imperfecta, hypercalcemia, hyperparathyroidism, osteomalacia, osteomalacia, osteomalacia, osteomalacia, osteomalacia, Paget's disease of bone, bone loss due to rheumatoid arthritis, inflammatory arthritis, osteomyelitis, corticosteroid treatment, metastatic bone diseases, periodontal bone loss, bone loss due to cancer, age-related loss of bone mass, other forms of osteopenia, as well as in instances where facilitation of bone repair or replacement is desired such as bone fractures, bone defects, plastic surgery, dental and other implantations.

[0134] Based on this discovery, the present invention further provides for a method of discovery of agents or compounds which modulate the Syk kinase pathway and/or the Vav3 pathway for use in modulating the differentiation and/or function of osteoclasts. Thus, in one embodiment, methods are provided for screening agents or compounds which inhibit Syk kinase or Vav3, thereby modulating the differentiation and/or function of osteoclasts.

[0135] In one embodiment, agents that interact with (*e.g.*, bind to) Syk kinase or Vav3, a Syk kinase or Vav3 fragment (*e.g.* a functionally active fragment), a Syk kinase-related polypeptide, a Vav3-related polypeptide, a fragment thereof, or a Syk kinase or Vav3 fusion protein are identified in a cell-based assay system. In accordance with this embodiment, cells expressing a Syk kinase or Vav3, a fragment

of a Syk kinase or Vav3, a Syk kinase -related polypeptide, a Vav3-related polypeptide, a fragment thereof, or a Syk kinase or Vav3 fusion protein are contacted with a candidate compound or a control compound and the ability of the candidate compound to interact with the Syk kinase and/or Vav3 is determined. Similarly, agents that modulate (*e.g.*, inhibit or activate) Syk kinase or Vav3, a Syk kinase or Vav3 fragment (*e.g.* a functionally active fragment), an Syk kinase-related polypeptide, a Vav3-related polypeptide, a fragment thereof, or a Syk kinase or Vav3 fusion protein are identified in a cell-based assay system. In this instance, the ability of a candidate compound to modulate the phosphorylation status of Syk kinase or Vav3, or alternatively to modulate the activity of a downstream target or intracellular protein in the Syk kinase pathway and/or Vav3 pathway is determined in a cell-based assay. If desired, this assay may be used to screen a plurality (*e.g.* a library) of candidate compounds. The cell, for example, can be of prokaryotic origin (*e.g.*, *E. coli*) or eukaryotic origin (*e.g.*, yeast, insect or mammalian). Further, the cells can express the Syk kinase or Vav3, fragment of the Syk kinase or Vav3, Syk kinase -related polypeptide, a Vav3-related polypeptide, a fragment thereof, or a Syk kinase or Vav3 fusion protein endogenously or be genetically engineered to express the Syk kinase or Vav3, fragment of the Syk kinase or Vav3, Syk kinase -related polypeptide, Vav3-related polypeptide, a fragment of the Syk kinase -related polypeptide, a fragment of the Vav3-related polypeptide, or a Syk kinase or Vav3 fusion protein. In some embodiments, the Syk kinase or Vav3, fragment of the Syk kinase or Vav3, etc. or the candidate compound is labeled, for example with a radioactive label (such as  $^{32}\text{P}$ ,  $^{35}\text{S}$  or  $^{125}\text{I}$ ) or a fluorescent label (such as fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde or fluorescamine) to enable detection of an interaction between a Syk kinase or Vav3 and a candidate compound and/or to enable detection of the phosphorylation of the Syk kinase or Vav3, etc. The ability of the candidate compound to interact directly or indirectly with a Syk kinase or Vav3, a fragment of a Syk kinase or Vav3, a Syk kinase -related polypeptide, a fragment of a Syk kinase-related polypeptide, a Vav3-related polypeptide, a fragment of a Vav3-related polypeptide, or a Syk kinase or Vav3 fusion protein or to modulate the activity of Syk kinase or Vav3, including the

phosphorylation or kinase activity thereof, can be determined by methods known to those of skill in the art. For example, the interaction or modulation by a candidate compound can be determined by flow cytometry, a scintillation assay, immunoprecipitation or western blot analysis, based on the present description.

[0136] The method includes the exposure of an osteoclast culture or osteoclast precursor culture to a candidate Syk or Vav3 modulator, and determining the duration and intensity of the response (for instance the differentiation and/or function of the osteoclast in culture) in the presence of the candidate and comparing the duration and intensity to that response in the absence of the candidate or in the presence of a known Syk and/or Vav3 inhibitor. The comparison step of the invention can be preferably performed directly, i.e., by comparing the culture's response to the candidate Syk or Vav3 modulator to that of a known Syk or Vav3 modulator in a contemporaneous parallel culture. Alternatively, the comparison can be made with an historical control showing elevation that is comparable to that observed under the same conditions with the culture and a known Syk or Vav3 modulator

[0137] In an alternative embodiment the comparison is performed longitudinally. Replicate cultures, i.e., at least duplicate, are established and the candidate compound is introduced into the cultures. The response of the cultures at time points that are shortly after the introduction and before and at or after some time (for instance one hour) following the introduction is determined. A Syk or Vav3 modulator can be identified by the persistence of the response by comparison to a contemporaneous control.

[0138] An exemplary method of screening osteogenic compounds, particularly compounds which modulate osteoclast differentiation and/or function, comprises selecting a compound that modulates, particularly inhibits, some portion of the Dap12/Syk pathway and/or the Vav3 pathway, performing an osteoclast assay with said compound and determining the result of the osteoclast assay, wherein a result indicates that the tested compound possesses osteogenic potential.

[0139] Briefly, selecting the compounds that interact with or bind to Syk or Vav3, other proteins in the Syk pathway or the Vav3 pathway, or otherwise inhibit or block the Syk pathway and/or Vav3 pathway, may be performed in multiple ways. The compounds may first be chosen based on their structural and functional characteristics, using one of a number of approaches known in the art. For instance, homology modeling can be used to screen small molecule libraries in order to determine which molecules would be candidates to interact with Syk or Vav3, thereby selecting plausible targets. See neogenesis.com for a commercially available screening of compounds using multiple different approaches such as an automated ligand identification system and quantized surface complementarity. The compounds to be screened can include both natural and synthetic ligands. Furthermore, any desired compound may be examined for its ability to interact with or bind to Syk or Vav3, including as described below.

[0140] Binding to or interaction with Syk or Vav3 or other proteins may be determined by performing an assay such as, e.g., a binding assay between a desired compound and Syk or Vav3. In one aspect, this is done by contacting said compound to Syk or Vav3 and determining its dissociation rate. Numerous possibilities for performing binding assays are well known in the art. The indication of a compound's ability to bind to Syk or Vav3 is determined, e.g., by a dissociation rate, and the correlation of binding activity and dissociation rates is well established in the art. For example, the assay may be performed by radio-labeling a reference compound, e.g. Syk or Vav3, with  $^{125}\text{I}$  or other suitable radioactive marker, and incubating it with Syk or Vav3. Test compounds are then added to these reactions in increasing concentrations. After optimal incubation, the Syk/compound or Vav3/compound complexes are separated, e.g., with chromatography columns, and evaluated for bound  $^{125}\text{I}$ -labeled peptide with a gamma ( $\gamma$ ) counter. The amount of the test compound necessary to inhibit 50% of the reference compound's binding is determined. These values are then normalized to the concentration of unlabeled reference compound's binding (relative inhibitory concentration (RIC))

$1 = \text{concentration}_{\text{test}} / \text{concentration}_{\text{reference}}$ ). A small  $\text{RIC}^{-1}$  value indicates strong relative binding, whereas a large  $\text{RIC}^{-1}$  value indicates weak relative binding. See, for example, Latek et al., Proc. Natl. Acad. Sci. USA, Vol. 97, No. 21, pp. 11460-11465, 2000.

[0141] A Syk or Vav3 mimic may be computationally evaluated and designed by means of a series of steps in which chemical groups or fragments are screened and selected for their ability to associate with the individual binding pockets or interface surfaces of the protein (e.g. Syk). One skilled in the art may employ one of several methods to screen chemical groups or fragments for their ability to associate with Syk or Vav3. This process may begin by visual inspection of, for example, the protein/protein interfaces or the binding site on a computer screen based on the available crystal complex coordinates of Syk or a Syk pathway protein or Vav3 or a Vav3 pathway protein, including a protein known to interact with Syk and/or Vav3. Selected fragments or chemical groups may then be positioned in a variety of orientations, or docked, at an individual surface of Syk or Vav3 that participates in a protein/protein interface or in the binding pocket. Docking may be accomplished using software such as QUANTA and SYBYL, followed by energy minimization and molecular dynamics with standard molecular mechanics forcefields, such as CHARMM and AMBER (AMBER, version 4.0 (Kollman, University of California at San Francisco © 1994); QUANTA/CHARMM (Molecular Simulations, Inc., Burlington, Mass., ©1994)). Specialized computer programs may also assist in the process of selecting fragments or chemical groups. These include: GRID (Goodford, 1985, J. Med. Chem. 28:849-857), available from Oxford University, Oxford, UK; MCSS (Miranker & Karplus, 1991, Proteins: Structure, Function and Genetics 11:29-34), available from Molecular Simulations, Burlington, Mass.; AUTODOCK (Goodsell & Olsen, 1990, Proteins: Structure, Function, and Genetics 8:195-202), available from Scripps Research Institute, La Jolla, Calif.; and DOCK (Kuntz et al., 1982, J. Mol. Biol. 161:269-288), available from University of California, San Francisco, Calif.

[0142] Once suitable chemical groups or fragments have been selected, they can be assembled into a single compound or inhibitor. Assembly may proceed by visual inspection of the relationship of the fragments to each other in the three-dimensional image displayed on a computer screen in relation to the structure coordinates thereof. This would be followed by manual model building using software such as QUANTA or SYBYL. Useful programs to aid one of skill in the art in connecting the individual chemical groups or fragments include: CAVEAT (Bartlett et al., 1989, 'CAVEAT: A Program to Facilitate the Structure-Derived Design of Biologically Active Molecules'. In *Molecular Recognition in Chemical and Biological Problems*, Special Pub., Royal Chem. Soc. 78:182-196), available from the University of California, Berkeley, CA; 3D Database systems such as MACCS-3D (MDL Information Systems, San Leandro, Calif.). This area is reviewed in Martin, 1992, *J. Med. Chem.* 35:2145-2154; and HOOK (available from Molecular Simulations, Burlington, Mass.). Instead of proceeding to build a Syk or Vav3 mimic, in a step-wise fashion one fragment or chemical group at a time, as described above, such compounds may be designed as a whole or 'de novo' using either an empty binding site or the surface of a protein that participates in protein/protein interactions or optionally including some portion(s) of a known activator(s). These methods include: LUDI (Bohm, 1992, *J. Comp. Aid. Molec. Design* 6:61-78), available from Molecular Simulations, Inc., San Diego, Calif.; LEGEND (Nishibata & Itai, 1991, *Tetrahedron* 47:8985), available from Molecular Simulations, Burlington, Mass.; and LeapFrog (available from Tripos, Inc., St. Louis, Mo.). Other molecular modeling techniques may also be employed in accordance with this invention. See, e.g., Cohen et al., 1990, *J. Med. Chem.* 33:883-894. See also, Navia & Murcko, 1992, *Current Opinions in Structural Biology* 2:202-210.

[0143] Once a compound has been designed by the above methods, the efficiency with which that compound may bind to or interact with Syk or Vav3 or other proteins may be tested and optimized by computational evaluation. Inhibitors may interact with the protein in more than one conformation that is similar in overall binding energy. In those cases, the deformation energy of binding is taken to be the difference

between the energy of the free compound and the average energy of the conformations observed when the inhibitor binds to the protein.

[0144] A compound selected or designed for binding to Syk or Vav3 or other proteins may be further computationally optimized so that in its bound state it would preferably lack repulsive electrostatic interaction with the target protein. Such non-complementary electrostatic interactions include repulsive charge-charge, dipole-dipole and charge-dipole interactions. Specifically, the sum of all electrostatic interactions between the inhibitor and the protein when the mimic is bound to it preferably make a neutral or favorable contribution to the enthalpy of binding. Specific computer software is available in the art to evaluate compound deformation energy and electrostatic interaction. Examples of programs designed for such uses include: Gaussian 92, revision C (Frisch, Gaussian, Inc., Pittsburgh, Pa. ©1992); AMBER, version 4.0 (Kollman, University of California at San Francisco © 1994); QUANTA/CHARMM (Molecular Simulations, Inc., Burlington, Mass., ©1994); and Insight II/Discover (Biosym Technologies Inc., San Diego, Calif., © 1994). These programs may be implemented, for instance, using a computer workstation, as are well-known in the art. Other hardware systems and software packages will be known to those skilled in the art.

[0145] Once a Syk or Vav3 modulating compound has been optimally designed, for example as described above, substitutions may then be made in some of its atoms or chemical groups in order to improve or modify its binding properties, or its pharmaceutical properties such as stability or toxicity. Generally, initial substitutions are conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. One of skill in the art will understand that substitutions known in the art to alter conformation should be avoided. Such altered chemical compounds may then be analyzed for efficiency of binding to Syk or Vav3 by the same computer methods described in detail above.

#### Performing Osteoclast and Bone Formation Assays and Determining Results

[0146] Once a compound that binds to Syk or Vav3 or otherwise modulates the Syk or Vav3 pathway is selected, it is then tested for its ability to modulate the differentiation or function of osteoclasts. There are multiple osteoclast culture systems or methods and bone formation assays that can be used successfully to screen potential osteogenic compounds of this invention. See, e.g., U.S. Pat. No. 6,080,779.

[0147] One osteoclast culture for use in screening is a neonatal mouse calvaria assay. Briefly, four days after birth, the front and parietal bones of neonatal mouse pups (e.g., ICR Swiss white mice) are removed by microdissection and split along the sagittal suture. The bones are then incubated in a specified medium, wherein the medium contains either test or control compounds. Following the incubation, the bones are removed from the media, and fixed in 10% buffered formalin, decalcified in EDTA, processed through graded alcohols, and embedded in paraffin wax. Sections of the calvaria are prepared and assessed using histomorphometric analysis of bone formation and bone resorption. Bone changes are measured on sections. Osteoblasts and osteoclasts are identified by their distinctive morphology.

[0148] In addition to this assay, the effect of compounds on murine calvarial bone growth can also be tested *in vivo*. In one such example of this screening assay, young male mice (e.g., ICR Swiss white mice), aged 4-6 weeks are employed, using 4-5 mice per group. Briefly, the test compound or the appropriate control is injected into subcutaneous tissue over the right calvaria of normal mice. The mice are sacrificed (after allowing for bone growth or loss to occur, e.g. on day 14), and net bone growth is measured by histomorphometric means. Bone samples are cleaned from adjacent tissues and fixed in 10% buffered formalin, decalcified, processed through graded alcohols, and embedded in paraffin wax. Sections of the calvaria are prepared, and representative sections are selected for histomorphometric assessment of the effects of bone formation and bone resorption. In one embodiment, sections are measured by using a camera lucida attachment to trace directly the microscopic image onto a digitizing plate. Bone changes are measured on sections over adjacent 1x1 mm fields

on both the injected and noninjected sides of calvaria. New bone may be identified by those skilled in the art by its characteristic tinctorial features, and osteoclasts and osteoblasts may be identified by their distinctive morphology or other suitable marker recognized by the skilled artisan. Histomorphometry software (OsteoMeasure, Osteometrix, Inc., Atlanta) can be used to process digitized input to determine cell counts and measure areas or perimeters.

[0149] Additional exemplary *in vivo* assays include dosing assays in intact animals, including dosing assays in acute ovariectomized (OVX) animals and assays in chronic OVX animals. Prototypical dosing in intact animals may be accomplished by subcutaneous, intraperitoneal or oral administration, and may be performed by injection, sustained release or other delivery techniques. The time period for administration of test compound may vary (for instance, 14 days, 28 days, as well as 35 days or longer may be appropriate).

[0150] As an example, *in vivo* oral or subcutaneous dosing assay may be performed as described: In a typical study, 70 three-month-old female Sprague-Dawley rats are weight-matched and divided into treatment groups, with at least several animals in each group. This includes a baseline control group of animals sacrificed at the initiation of the study; a control group administered vehicle only; a PBS or saline-treated control group; and a positive group administered a compound known to enhance net bone formation. Three dosage levels of the test compound are administered to the remaining groups. Test compound, saline, and vehicle are administered (e.g. once per day) for a number of days (for instance at least 14 days, 28 days, or 35 days – wherein an effect is expected in the positive group). All animals are injected calcein nine days and two days before sacrifice (to ensure proper labeling of newly formed bone). Weekly body weights are determined. At the end of the period of compound administration, the animals are weighed and bled by orbital or cardiac puncture. Serum calcium, phosphate, osteocalcin, and CBCs are determined. Both leg bones (femur and tibia) and lumbar vertebrae are removed, cleaned of adhering soft tissue, and stored in 70% ethanol or 10% formalin for evaluation, for

instance as performed by peripheral quantitative computed tomography (pQCT; Ferretti, J, Bone, 17: 353S-364S, 1995), dual energy X-ray absorptiometry (DEXA; Laval-Jeantet A. et al., Calcif Tissue Intl, 56:14-18, 1995, and Casez J. et al., Bone and Mineral, 26:61-68, 1994) and/or histomorphometry. The effect of test compounds on bone remodeling or net bone formation, including bone loss and osteoclast function can thus be evaluated.

[0151] Test compounds can also be assayed in acute ovariectomized animals. Such assays may also include an estrogen-treated group as a control. An example of the test in these animals is briefly described: In a typical study, 80 three-month-old female Sprague-Dawley rats are weight-matched and divided into treatment groups, with at least several animals in each group. This includes a baseline control group of animals sacrificed at the initiation of the study; three control groups (sham OVX and vehicle only, OVX and vehicle only, and OVX and PBS only); and a control OVX group that is administered a compound known to block or reduce bone resorption or enhance bone formation (including an anti-resorptive or anabolic compound). Different dosage levels of the test compound are administered to remaining groups of OVX animals.

[0152] Since ovariectomy induces hyperphagia, all OVX animals are pair-fed with sham OVX animals throughout the study. Test compound, positive control compound, PBS or saline or vehicle alone is administered orally or subcutaneously (e.g., once per day) for the treatment period. As an alternative, test compounds can be formulated in implantable pellets that are implanted, or may be administered orally, such as by gastric gavage. All animals are injected with calcein nine days and two days before sacrifice. Weekly body weights are determined. At the end of the treatment cycle, the animals blood and tissues are processed as described above.

[0153] Test compounds may also be assayed in chronic OVX animals. Briefly, six month old female, Sprague-Dawley rats are subjected to sham surgery (sham OVX), or ovariectomy (OVX) at the beginning of the experiment, and animals are sacrificed at the same time to serve as baseline controls. Body weights are monitored weekly.

After approximately six weeks or more of bone depletion, sham OVX and OVX rats are randomly selected for sacrifice as depletion period controls. Of the remaining animals, 10 sham OVX and 10 OVX rats are used as placebo-treated controls. The remaining animals are treated with 3 to 5 doses of test compound for a period of 35 days. As a positive control, a group of OVX rats can be treated with a known anabolic or anti-resorptive agent in this model, such as bisphosphonate, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, a supplemental bone formation agent parathyroid hormone (PTH) or its derivative (Kimmel et al., *Endocrinology*, 132: 1577-1584, 1993), PTHRP, a bone morphogenetic protein, osteogenin, NaF, PGE<sub>2</sub> agonists, a statin, and a RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomerized form of RANKL. At the end of the experiment, the animals are sacrificed and femurs, tibiae, and lumbar vertebrae to 4 are excised and collected. The proximal left and right tibiae are used for pQCT measurements, cancellous bone mineral density (BMD), and histology, while the midshaft of each tibiae is subjected to cortical BMD or histology. The femurs are prepared for pQCT scanning of the midshaft prior to biomechanical testing. With respect to lumbar vertebrae (LV), LV2 are processed for BMD (pQCT may also be performed), LV3 are prepared for undecalcified bone histology, and LV4 are processed for mechanical testing.

[0154] In addition, osteoclast cultures, containing macrophages, osteoclast precursors and osteoclasts, can be generated from bone marrow precursors, particularly from bone marrow macrophages and utilized in assessment of compounds for osteoclast modulating activity. Bone marrow macrophages are cultured in 48- or 96-well cell culture dishes in the presence of M-CSF (10ng/ml), RANKL (100ng/ml), with or without addition of compound(s) or control(s), and medium changed (e.g. on day 3). Osteoclast-like cells are characterized by staining for tartrate-resistant acid phosphatase (TRAP) activity. In assessing bone resorption, for instance using a pit assay, osteoclasts are generated on whale dentin slices from bone marrow macrophages. After three days of culture to generate osteoclasts, compound(s) or control(s) are added to the culture for two days. At the end of the experiment, cells

are TRAP stained and photographed to document cell number. Cells are then removed from the dentin slices with 0.5M ammonium hydroxide and mechanical agitation. Maximum resorption lacunae depth is measured using a confocal microscope (Microradiance, Bio-Rad Laboratories, Hercules, CA). For evaluation of pit number and resorbed area, dentin slices are stained with Coumassie brilliant blue and analyzed with light microscopy using Osteomeasure software (Osteometrics, Decatur, Georgia) for quantitation.

[0155] In a preferred embodiment, osteoclast modulating ability of a compound is tested in an *in vitro* assay utilizing osteoclasts, osteoclast precursor cells or osteoclast-like cells. General protocols for treatment of osteoclasts with a compound are well established and known in the art. For instance, bone marrow macrophages may be utilized to generate osteoclasts in vitro as described herein. It is to be noted that the conditions used will vary according to the cell lines and compound used, their respective amounts, and additional factors such as plating conditions and media composition. Such adjustments are readily determined by one skilled in this art.

[0156] The function of osteoclasts or differentiation of osteoclast precursors may be determined by assessing the activation of intracellular proteins indicative of osteoclast differentiation and/or function, particularly including, but not limited to, proteins or kinases such as phospholipase C $\gamma$ 1 (PLC $\gamma$ ), VAV, CBL, ERK and JNK which may be substrates for Syk or activated or more active in the presence of phosphorylated Syk, or proteins or kinases such as Rho and Rac which may be substrates for Vav3 or activated or more active in the presence of phosphorylated Vav3. In addition, the activation of Syk or Vav3 or phosphorylation of Syk or Vav3 may be assessed. Thus, following the incubation of osteoclasts with a test compound, the cells are lysed and their intracellular contents subjected to the appropriate tests, such as Western blots, kinase assays, and electrophoretic mobility gel shift assays (EMSAs).

[0157] A Western blot can be generally performed as follows. Once the cell lysates are generated, the intracellular proteins are separated on the basis of size by utilizing

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The separated proteins are transferred by electroblotting to a suitable membrane (such as nitrocellulose or polyvinylidene fluoride) to which they adhere. The membrane is washed to reduce non-specific signals, and then probed with an antibody which recognizes only the specific amino acid which has been phosphorylated as a result of Syk or Vav3 phosphorylation and/or signaling. After further washing, which removes excess antibody, a second antibody, which recognizes the first antibody (bound to specifically-phosphorylated proteins on the membrane) and contains a reporter moiety is applied to the membrane. The addition of a developing agent, which interacts with a reporter moiety on the second antibody results in visualization of the bands.

[0158] A kinase assay, for example for ERK1/2, can be performed by utilizing a known substrate for this kinase such as p90 ribosomal S6 protein kinase (RSK). Briefly, by way of example, treated osteoclasts are washed in ice-cold PBS and extracted with lysis buffer in order to obtain cell lysates. Supernatants obtained after microcentrifugation of cell lysates are incubated with goat anti-RSK2 antibody (1:200) together with protein G-Sepharose at 4°C overnight. The beads are collected by microcentrifugation, washed twice with lysis buffer, followed by kinase buffer. RSK2 phosphotransferase activity in the beads is measured by using S6 kinase assay kit and [<sup>32</sup>P]ATP according to the protocols provided by the manufacturer (Upstate Biotechnology, Inc).

[0159] An additional assay that can be applied to determine differentiation and/or function of osteoclasts is an electrophoretic mobility gel shift assay (EMSA). Briefly, an EMSA may be conducted as follows. Nuclei of treated osteoclasts are isolated and their extracts generated. The nuclear proteins are then incubated with a specific oligonucleotide probe that has been labeled <sup>32</sup>P orthophosphate. After an appropriate time, the putative protein-DNA complexes are separated on a PAGE gel (no SDS present), which is dried and exposed to an X-ray film. If a specific complex has formed a band will be visible on the developed film. Typically, appropriate controls are run in parallel with the experimental sample(s) in order to ensure that the band is

specific for activated osteoblasts. For detailed procedures on Western blotting, kinase assays, and EMSA (see for example Lai et al. (2001) J Biol Chem 276(17):14443-14450). Additionally, cell-based assays for osteoclast differentiation and function, based on measuring or visualizing F-actin to detect the actin ring or by visualizing resorption pits by hematoxylin staining in bone marrow macrophage derived osteoclasts. On addition, proliferation of osteoclasts and osteoclast precursors in cultures of bone marrow macrophages may be assessed, e.g. by BrdU incorporation. These assays are well known in the art and easily performed by a skilled artisan.

[0160] In one embodiment, the method of screening osteoclast modulating compounds involves incubating a test compound with osteoclasts, osteoclast precursor cells or osteoclast-like cells under conditions sufficient for such incubation. The test compound may be a compound that binds to Syk or Vav3. Selecting the compounds that bind to Syk or Vav3 may be performed in multiple ways as described above and herein. The compound may also not bind to Syk or Vav3, but may inhibit Syk or Vav3, or inhibit one or more of the proteins in the Syk pathway downstream of Syk or in the Vav3 pathway downstream of Vav3.

[0161] General protocols and assays for the treatment of osteoclasts with a compound are known to the skilled artisan and are described herein. Similarly, modulation of osteoclast differentiation and/or function and/or intracellular compounds in the Syk or Vav3 pathway related to osteoclast differentiation and/or function, may be performed as described above. For purposes of the present embodiment, the assays may consist of determining the activation of intracellular proteins correlated with osteoclast differentiation and/or function. These proteins include but are not limited phospholipase C $\gamma$ 1 (PLC $\gamma$ ), VAV, CBL, ERK and JNK which may be substrates for Syk or activated or more active in the presence of phosphorylated Syk and Rho and Rac which may be substrates for Vav3 or activated or more active in the presence of phosphorylated or activated Vav3.

[0162] In a preferred embodiment, the modulation comprises phosphorylation of

intracellular proteins in the Syk and/or Vav3 pathway, and more preferably of kinases, particularly including Syk kinase and Vav3. The methods of the present invention may utilize any of the appropriate assays available in the art for determining whether a kinase has been phosphorylated. Preferably, the assays used are Western blots or kinase assays.

[0163] In an embodiment, methods for screening osteogenic compounds based on their ability to activate phosphatase(s) (partially or completely) are also provided herein. The compounds to be screened may include compounds that bind to Syk or Vav3 or dephosphorylate Syk or Vav3, and methods for selecting such compounds are described above. The phosphatases inhibit the kinases specific for osteoclast differentiation and/or function, including Syk and Vav3 and proteins (kinases) in the Syk or Vav3 pathway, for instance phospholipase C $\gamma$ 1 (PLC $\gamma$ ), VAV, CBL, ERK and JNK which may be substrates for Syk or activated or more active in the presence of phosphorylated Syk and Rho and Rac which may be substrates for Vav3 or activated or more active in the presence of phosphorylated or activated Vav3. Preferably, the phosphatases are Syk specific or Vav3 specific or Syk pathway or Vav3 pathway specific. While not being bound to a particular theory, this method is feasible for this purpose due to the fact that in some instances a kinase activity is tightly regulated by its corresponding phosphatase. In case of ERK1/2, the phosphatases are known as the mitogen activated protein kinases phosphatase-1,2,3 (MKP-1,2,3). These phosphatases belong to a family of dual specificity phosphatases, which are responsible for the removal of phosphate groups from the threonine and tyrosine residues on their corresponding kinases (Camps et al., FASEB J., 14, pp. 6-16, 1999). The prompt removal of phosphate groups by phosphatases ensures that kinase activation is short-lived and that the level of phosphorylation is low in a resting cell. However, in order for the phosphatase to be active and remove phosphate groups, it also needs to be phosphorylated. Therefore, activation of phosphatase activity results in inactivation of kinase activity.

[0164] The ability of the test compounds to activate phosphatase(s) can be determined

by performing Western blots or kinase assays. See above. For additional details on assessing phosphatase activity, see Muda et al., J Biol Chem., 273:9323-9329, 1998, and Camps et al., Science 280:1262-1265, 1998. If the compound is determined to possess enhanced phosphatase activity, it can further be tested in one of the bone formation or osteoclast assays to determine its effect on osteoclast differentiation and/or activity.

#### Candidate Compounds and Agents

[0165] Examples of agents, candidate compounds or test compounds include, but are not limited to, nucleic acids (*e.g.*, DNA and RNA), carbohydrates, lipids, proteins, peptides, peptidomimetics, small molecules and other drugs. In one preferred aspect, agents can be obtained using any of the numerous suitable approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997, Anticancer Drug Des. 12:145; U.S. Patent No. 5,738,996; and U.S. Patent No. 5,807,683).

[0166] Phage display libraries may be used to screen potential ligands or Syk or Vav3 modulators. Their usefulness lies in the ability to screen, for example, a library displaying a billion different compounds with only a modest investment of time, money, and resources. For use of phage display libraries in a screening process, see, for instance, Kay et al., Methods, 240-246, 2001. An exemplary scheme for using phage display libraries to identify compounds that bind or interact with Syk or Vav3 may be described as follows: initially, an aliquot of the library is introduced into microtiter plate wells that have previously been coated with target protein, *e.g.* Syk or Vav3. After incubation (*e.g.* 2 hrs), the nonbinding phage are washed away, and the

bound phage are recovered by denaturing or destroying the target with exposure to harsh conditions such as, for instance pH 2, but leaving the phage intact. After transferring the phage to another tube, the conditions are neutralized, followed by infection of bacteria with the phage and production of more phage particles. The amplified phage are then rescreened to complete one cycle of affinity selection. After three or more rounds of screening, the phage are plated out such that there are individual plaques that can be further analyzed. For example, the conformation of binding activity of affinity-purified phage for Syk or Vav3 may be obtained by performing ELISAs. One skilled in the art can easily perform these experiments. In one aspect, a Syk or Vav3 molecule used for any of the assays may be selected from a recombinant Syk or Vav3 protein, a Syk or Vav3 fusion protein, an analog, derivative, or mimic thereof. In a preferred aspect, Syk is a recombinant Syk protein and Vav3 is a recombinant protein.

[0167] Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al., 1993, Proc. Natl. Acad. Sci. USA 90:6909; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422; Zuckermann et al., 1994, J. Med. Chem. 37:2678; Cho et al., 1993, Science 261:1303; Carrell et al., 1994, Angew. Chem. Int. Ed. Engl. 33:2059; Carrell et al., 1994, Angew. Chem. Int. Ed. Engl. 33:2061; and Gallop et al., 1994, J. Med. Chem. 37:1233.

[0168] Libraries of compounds may be presented, *e.g.*, presented in solution (*e.g.*, Houghten, 1992, Bio/Techniques 13:412-421), or on beads (Lam, 1991, Nature 354:82-84), chips (Fodor, 1993, Nature 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull et al., 1992, Proc. Natl. Acad. Sci. USA 89:1865-1869) or phage (Scott and Smith, 1990, Science 249:386-390; Devlin, 1990, Science 249:404-406; Cwirla et al., 1990, Proc. Natl. Acad. Sci. USA 87:6378-6382; and Felici, 1991, J. Mol. Biol. 222:301-310).

[0169] The methods of screening compounds may also include the specific

identification or characterization of such compounds, whose osteoclast modulating potential was determined by the methods described above. If the identity of the compound is known from the start of the experiment, no additional assays are needed to determine its identity. However, if the screening for compounds that modulate Syk or Vav3 is done with a library of compounds, it may be necessary to perform additional tests to positively identify a compound that satisfies all required conditions of the screening process. There are multiple ways to determine the identity of the compound. One process involves mass spectrometry, for which various methods are available and known to the skilled artisan (see for instance neogenesis.com). Neogenesis' ALIS (automated ligand identification system) spectral search engine and data analysis software allow for a highly specific identification of a ligand structure based on the exact mass of the ligand. One skilled in the art can also readily perform mass spectrometry experiments to determine the identity of the compound.

**[0170]** Antibodies, including polyclonal and monoclonal antibodies, particularly anti-Syk or anti-Vav3 antibodies and neutralizing antibodies may be useful as compounds to modulate osteoclast differentiation and/or function. Also, antibodies including both polyclonal and monoclonal antibodies, and drugs that modulate the activity of Syk kinase or Vav3 and/or their subunits may possess certain diagnostic applications and may for example, be utilized for the purpose of detecting and/or measuring conditions such as bone diseases, bone loss, or osteoclast differentiation and/or function. Syk kinase or Vav3 or its subunits may be used to produce both polyclonal and monoclonal antibodies to themselves in a variety of cellular media, by known techniques such as the hybridoma technique utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. Likewise, small molecules that mimic or antagonize the activity(ies) of Syk kinase or Vav3 may be discovered or synthesized, and may be used in diagnostic and/or therapeutic protocols.

#### Compositions and Methods of Using Syk and/or Vav3 Pathway Compounds

[0171] In a preferred embodiment of the invention, a method of preventing or inhibiting bone loss or of enhancing bone formation is provided by administering compositions comprising compounds identified by the screening methods provided herein. The Syk and/or Vav3 modulating compositions of the present invention may be utilized by providing an effective amount of such compositions to a subject in need thereof.

[0172] The present invention further contemplates therapeutic compositions useful in practicing the therapeutic methods of this invention. A subject therapeutic composition includes, in admixture, a pharmaceutically acceptable excipient (carrier) and one or more of a Syk kinase or Vav3 modulator, polypeptide analog thereof or fragment thereof, as described herein as an active ingredient. In a preferred embodiment, the composition comprises one or more compound or agent capable of blocking or inhibiting Syk kinase or Vav3 or the Syk kinase or Vav3 pathway.

[0173] In general, for use in treatment, the compounds of the invention may be used alone or in combination with each other (i.e. one or more of a Syk or Vav3 modulator), with or without other compositions for the treatment of bone loss. Such compositions include anti-resorptives and anabolic agents, such as, but not limited to a bisphosphonate, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, or a supplemental bone formation agent like parathyroid hormone (PTH) or its derivative or fragments thereof, PTH related protein (PTHrp), a bone morphogenetic protein, osteogenin, NaF, a PGE<sub>2</sub> agonist, a statin (see U.S. Pat. No. 6,080,779 incorporated herein by reference) or one or more RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomeric form of RANKL. Compositions comprising one or more Syk modulator and one or more other anti-resorptive or anabolic agent are provided and included in the invention. In a particular embodiment, compositions comprising one or more Syk modulator and one or more more RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomeric form of RANKL, are provided.

[0174] The compounds or compositions of the invention may be combined for administration with or embedded in polymeric carrier(s), biodegradable or biomimetic matrices or in a scaffold. The carrier, matrix or scaffold may be of any material that will allow composition to be incorporated and expressed and will be compatible with the addition of cells or in the presence of cells. Preferably, the carrier matrix or scaffold is predominantly non-immunogenic and is biodegradable. Examples of biodegradable materials include, but are not limited to, polyglycolic acid (PGA), polylactic acid (PLA), hyaluronic acid, catgut suture material, gelatin, cellulose, nitrocellulose, collagen, albumin, fibrin, alginate, cotton, or other naturally-occurring biodegradable materials. It may be preferable to sterilize the matrix or scaffold material prior to administration or implantation, e.g., by treatment with ethylene oxide or by gamma irradiation or irradiation with an electron beam. In addition, a number of other materials may be used to form the scaffold or framework structure, including but not limited to: nylon (polyamides), dacron (polyesters), polystyrene, polypropylene, polyacrylates, polyvinyl compounds (e.g., polyvinylchloride), polycarbonate (PVC), polytetrafluorethylene (PTFE, teflon), thermanox (TPX), polymers of hydroxy acids such as polylactic acid (PLA), polyglycolic acid (PGA), and polylactic acid-glycolic acid (PLGA), polyorthoesters, polyanhydrides, polyphosphazenes, and a variety of polyhydroxyalkanoates, and combinations thereof. Matrices suitable include a polymeric mesh or sponge and a polymeric hydrogel. In the preferred embodiment, the matrix is biodegradable over a time period of less than a year, more preferably less than six months, most preferably over two to ten weeks. The polymer composition, as well as method of manufacture, can be used to determine the rate of degradation. For example, mixing increasing amounts of polylactic acid with polyglycolic acid decreases the degradation time. Meshes of polyglycolic acid that can be used can be obtained commercially, for instance, from surgical supply companies (e.g., Ethicon, N.J.). A hydrogel is defined as a substance formed when an organic polymer (natural or synthetic) is cross-linked via covalent, ionic, or hydrogen bonds to create a three-dimensional open-lattice structure which entraps water molecules to form a gel. In general, these polymers are at least partially

soluble in aqueous solutions, such as water, buffered salt solutions, or aqueous alcohol solutions, that have charged side groups, or a monovalent ionic salt thereof.

[0175] For use in treatment of animal subjects, the compositions of the invention can be formulated as pharmaceutical or veterinary compositions. Depending on the subject to be treated, the mode of administration, and the type of treatment desired, e.g., prevention, prophylaxis, therapy; the compositions are formulated in ways consonant with these parameters. A summary of such techniques is found in Remington's Pharmaceutical Sciences, latest edition, Mack Publishing Co., Easton, Pa.

[0176] The preparation of therapeutic compositions which contain polypeptides, analogs or active fragments as active ingredients is well understood in the art. Formulations may be prepared in a manner suitable for systemic administration or for topical or local administration. Systemic formulations include, but are not limited to those designed for injection (e.g., intramuscular, intravenous or subcutaneous injection) or may be prepared for transdermal, transmucosal, nasal, or oral administration. Such compositions may be prepared as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. The formulation will generally include a diluent as well as, in some cases, adjuvants, buffers, preservatives and the like. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents which enhance the effectiveness of the active ingredient.

[0177] A polypeptide, analog or active fragment can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like. For oral administration, the compositions can be administered also in liposomal compositions or as microemulsions. Suitable forms include syrups, capsules, tablets, as is understood in the art.

[0178] The compositions of the present invention may also be administered locally to sites in subjects, both human and other vertebrates, such as domestic animals, rodents and livestock, where bone formation and growth are desired using a variety of techniques known to those skilled in the art. For example, these may include sprays, lotions, gels or other vehicles such as alcohols, polyglycols, esters, oils and silicones. Such local applications include, for example, at a site of a bone fracture or defect to repair or replace damaged bone. Additionally, a bone modulating composition may be administered e.g., in a suitable carrier, at a junction of an autograft, allograft or prosthesis and native bone to assist in binding of the graft or prosthesis to the native bone.

[0179] The administration of the compositions of the present invention may be pharmacokinetically and pharmacodynamically controlled by calibrating various parameters of administration, including the frequency, dosage, duration mode and route of administration. Thus, in one embodiment bone mass formation is achieved by administering a bone forming composition in a non-continuous, intermittent manner, such as by daily injection and/or ingestion. Variations in the dosage, duration and mode of administration may also be manipulated to produce the activity required.

[0180] The therapeutic Syk or Vav3 modulator compositions are conventionally administered in the form of a unit dose, for instance intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

[0181] The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of inhibition of Syk or Vav3 desired or the extent or severity of bone disease. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.01 to 10, preferably about 0.01 to 0.1, preferably about 0.01 to 0.5, preferably about 0.1 to 0.5, preferably about 0.5 to about 10, and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. However, dosage levels are highly dependent on the nature of the disease or situation, the condition of the subject, the judgment of the practitioner, and the frequency and mode of administration. If the oral route is employed, the absorption of the substance will be a factor effecting bioavailability. A low absorption will have the effect that in the gastro-intestinal tract higher concentrations, and thus higher dosages, will be necessary. Suitable regimes for initial administration and further administration are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain desired concentrations, e.g. in the blood, are contemplated.

[0182] It will be understood that the appropriate dosage of the substance should suitably be assessed by performing animal model tests, wherein the effective dose level (e.g. ED<sub>50</sub>) and the toxic dose level (e.g. TD<sub>50</sub>) as well as the lethal dose level (e.g. LD<sub>50</sub> or LD<sub>10</sub>) are established in suitable and acceptable animal models. Further, if a substance has proven efficient in such animal tests, controlled clinical trials should be performed.

[0183] The compound or composition of the present invention may be modified or formulated for administration at the site of bone or to bone cells, particularly osteoclasts. Such modification may include, for instance, formulation which facilitate or prolong the half-life of the compound or composition, particularly in the osteoclast environment. Additionally, such modification may include the formulation of a compound or composition to include a targeting protein or sequence which facilitates or enhances the uptake of the compound/composition to bone or bone precursor cells. In a particular embodiment, such modification results in the preferential targeting of the compound to bone or bone precursor cells versus other locations or cells. In one embodiment, a tetracycline, tetracycline family or bisphosphonate may be utilized to target the compound or composition of the present invention to bone or bone cells, including osteoclasts and osteoclast precursors. Bone targeted pyrido[2,3-d]pyrimidin-7-ones have been described as potent inhibitors of Src tyrosine kinase and as anti-resorptive agents (Vu CB et al (2003) Bioorg Med Chem Lett 13(18):3071-3074). Novel heterocycles as bone targeting compounds are disclosed in U.S. Patent Publication No. 2002/0103161A1, which is incorporated herein by reference in its entirety.

#### Assays and Test Kits

[0184] In a further embodiment of this invention, commercial test kits suitable for use by a medical specialist may be prepared to determine the presence or absence of or the amount of Syk kinase or Vav3 or Syk kinase activity or Vav3 activity in cells. Accordingly, one class of such kits will contain at least the labeled Syk or Vav3 or its

binding partner, for instance an antibody specific thereto, and directions, of course, depending upon the method selected, e.g., competitive, sandwich, and the like, all and any of which are methods well known to the skilled artisan. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

**[0185]** Accordingly, a test kit may be prepared for the demonstration of the presence or capability of cells for Syk activity, comprising:

- (a) a predetermined amount of at least one labeled immunochemically reactive component obtained by the direct or indirect attachment of Syk or Dap12 or a specific binding partner thereto, to a detectable label;

- (b) other reagents; and

- (c) directions for use of said kit.

**[0186]** More specifically, the diagnostic test kit may comprise:

- (a) a known amount of the Syk kinase as described above (or a binding partner) generally bound to a solid phase to form an immunosorbent, or in the alternative, bound to a suitable tag, or plural such end products, etc. (or their binding partners) one of each;

- (b) if necessary, other reagents; and

- (c) directions for use of said test kit.

**[0187]** In a further variation, the test kit may be prepared and used for the purposes stated above, which operates according to a predetermined protocol (e.g. competitive, sandwich, double antibody, etc.), and comprises:

- (a) a labeled component which has been obtained by coupling Syk to a detectable label;

- (b) one or more additional immunochemical reagents of which at least one reagent is a ligand or an immobilized ligand, which ligand is selected from the group consisting of:

- (i) a ligand capable of binding with the labeled component (a);

- (ii) a ligand capable of binding with a binding partner of the labeled component (a);
- (iii) a ligand capable of binding with at least one of the component(s) to be determined; and
- (iv) a ligand capable of binding with at least one of the binding partners of at least one of the component(s) to be determined; and
- (c) directions for the performance of a protocol for the detection and/or determination of one or more components of an immunochemical reaction between Syk and a specific binding partner thereto.

**[0188]** Accordingly, a test kit may be prepared for the demonstration of the presence or capability of cells for Vav3 activity, comprising:

- (a) a predetermined amount of at least one labeled immunochemically reactive component obtained by the direct or indirect attachment of Vav3 or a specific binding partner thereto, to a detectable label;
- (b) other reagents; and
- (c) directions for use of said kit.

**[0189]** More specifically, the diagnostic test kit may comprise:

- (a) a known amount of the Vav3 as described above (or a binding partner) generally bound to a solid phase to form an immunosorbent, or in the alternative, bound to a suitable tag, or plural such end products, etc. (or their binding partners) one of each;
- (b) if necessary, other reagents; and
- (c) directions for use of said test kit.

**[0190]** In a further variation, the test kit may be prepared and used for the purposes stated above, which operates according to a predetermined protocol (e.g. competitive, sandwich, double antibody, etc.), and comprises:

- (a) a labeled component which has been obtained by coupling Vav3 to a detectable label;

(b) one or more additional immunochemical reagents of which at least one reagent is a ligand or an immobilized ligand, which ligand is selected from the group consisting of:

- (i) a ligand capable of binding with the labeled component (a);
- (ii) a ligand capable of binding with a binding partner of the labeled component (a);
- (iii) a ligand capable of binding with at least one of the component(s) to be determined; and
- (iv) a ligand capable of binding with at least one of the binding partners of at least one of the component(s) to be determined; and

(c) directions for the performance of a protocol for the detection and/or determination of one or more components of an immunochemical reaction between Vav3 and a specific binding partner thereto.

**[0191]** The present invention extends to the preparation of antisense oligonucleotides and ribozymes that may be used to interfere with the expression of Syk kinase or Vav3 at the translational level. This approach utilizes antisense nucleic acid and ribozymes to block translation of a specific mRNA, either by masking that mRNA with an antisense nucleic acid or cleaving it with a ribozyme.

**[0192]** Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule. (See Weintraub, 1990; Marcus-Sekura, 1988.) In the cell, they hybridize to that mRNA, forming a double stranded molecule. The cell does not translate an mRNA in this double-stranded form. Therefore, antisense nucleic acids interfere with the expression of mRNA into protein. Oligomers of about fifteen nucleotides and molecules that hybridize to the AUG initiation codon will be particularly efficient, since they are easy to synthesize and are likely to pose fewer problems than larger molecules when introducing them into Syk-producing cells. Antisense methods have been used to inhibit the expression of many genes *in vitro* (Marcus-Sekura, 1988; Hambor et al., 1988).

[0193] Ribozymes are RNA molecules possessing the ability to specifically cleave other single stranded RNA molecules in a manner somewhat analogous to DNA restriction endonucleases. Ribozymes were discovered from the observation that certain mRNAs have the ability to excise their own introns. By modifying the nucleotide sequence of these RNAs, researchers have been able to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it (Cech, 1988.). Because they are sequence-specific, only mRNAs with particular sequences are inactivated.

[0194] Investigators have identified two types of ribozymes, *Tetrahymena*-type and "hammerhead"-type. (Hasselhoff and Gerlach, 1988) *Tetrahymena*-type ribozymes recognize four-base sequences, while "hammerhead"-type recognize eleven- to eighteen-base sequences. The longer the recognition sequence, the more likely it is to occur exclusively in the target mRNA species. Therefore, hammerhead-type ribozymes are preferable to *Tetrahymena*-type ribozymes for inactivating a specific mRNA species, and eighteen base recognition sequences are preferable to shorter recognition sequences.

[0195] Nucleic acid sequences encoding the Syk kinase or Vav3, including any transcribed portion thereof, may thus be used to prepare antisense molecules against, and ribozymes that cleave mRNAs for the Syk kinase or Vav3.

[0196] The use of RNA interference strategies to inhibit the expression of Syk kinase or Vav3 is further embodied in the invention. Thus, methods of RNA interference and small interfering RNA compositions are included in the methods and compositions of the present invention. RNA interference refers to the silencing of genes specifically by double stranded RNA (dsRNA) (Fine, A. et al (1998) Nature 391:806-811). In one embodiment, short or small interfering RNA (siRNA) is utilized (Elbashir, S.M. et al (2001) Nature 411:494-498). In addition, long double stranded RNA hairpins may be employed (Tavernarakis, N. et al (2000) Nature Genet 24:180-183; Chuang, C.F. and Meyerowitz, E.M. (2000) PNAS USA 97:4985-90;

Smith, NA et al (2000) Nature 407:319-20). Virus-mediated RNA interference against K-Ras has been described (B rummelkamp, T.R. et al (2002) Cancer Cell 2:243-247).

[0197] The invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention. The following examples are presented in order to more fully illustrate the preferred embodiments of the invention and should in no way be construed, however, as limiting the broad scope of the invention.

#### EXAMPLE 1

##### GST-RANKL AND OTHER RANKL OLOGOMERS RAPIDLY ACTIVATE MAP KINASES IN MURINE OSTEOCLAST PRECURSORS

[0198] Wild type C57BL/6 mice were purchased from Harlan Industries (Indianapolis, Ind.). For the isolation of osteoclast precursors, bone marrow macrophages (BMMs) were isolated from whole bone marrow of four to six week old mice and incubated in tissue culture dishes at 37°C in 5% CO<sub>2</sub>. After 24 hours in culture, the non-adherent cells were collected and layered on a Ficoll Hypaque gradient and the cells at the gradient interface were collected. Cells were replated at 65,000/cm<sup>2</sup> in  $\alpha$ -minimal essential medium, supplemented with 10% heat inactivated fetal bovine serum, at 37°C in 5% CO<sub>2</sub> in the presence of recombinant mouse M-CSF (10 ng/ml).

[0199] Purified GST-RANKL was obtained as outlined briefly below. CDNA encoding murine RANKL residues 158-316 was cloned into pGEX-4T-1 (Amersham, GenBank Accession No. U13853) downstream of glutathione S-transferase (GST), sequence. Following IPTG-mediated induction of protein expression in BL21 (DE3) Escherichia coli (Invitrogen), cells were lysed and GST-RANKL fusion protein was affinity purified from the cell lysates incubated with glutathione sepharose (Amersham). GST-RANKL was eluted from the affinity column, subjected to ion

exchange chromatography, and dialyzed against physiologic salt and pH. Purified GST-RANKL was then assayed for endotoxin contamination by limulus amoebocyte lysate assay, and quantitated for bioactivity by an in vitro osteoclastogenesis readout.

[0200] Cells were treated with GST-RANKL on day 4 or 5. In the experiments addressing the activation of Akt (see Example 2 below), the cells were cultured in serum and M-CSF free medium for 24 hours prior to GST-RANKL stimulation.

[0201] Immunoblotting (Western blotting) of osteoclast precursors was performed according to the following instructions. Cytokine-treated or control monolayers of BMMs were washed twice with ice-cold PBS. Cells were lysed in the buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{PO}_4$ , 1 mM NaF, and 1 times protease inhibitor cocktail. Fifty  $\mu\text{g}$  of cell lysates were boiled in the presence of SDS sample buffer (0.5 M Tris-HCl, pH 6.8, 10% w/v SDS, 10% glycerol, 0.05% w/v bromophenol blue) for 5 minutes and separated on SDS-PAGE, using 8% gels. Proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad, Richmond, Calif.) and incubated in blocking solution (5% non-fat dry milk in tris-buffered saline containing 0.1% Tween 20) for 1 hour to reduce nonspecific binding. Membranes were then exposed to primary antibodies overnight at 4°C, washed three times, and incubated with secondary goat anti-mouse or rabbit IgG horseradish peroxidase-conjugated antibody for 1 hour. Membranes were washed extensively, and enhanced chemiluminescence detection assay was performed following the manufacturer's directions (Amersham).

[0202] The results of the immunoblotting assay are depicted in FIGURE 1. As can be seen from this figure, the total cellular amounts of JNK, p38, and ERK did not change significantly at any point of the assay. The phosphorylation (activation) of ERK and p38 was detected 5 minutes following the GST-RANKL stimulation, peaked at 10 minutes after RANK/GST-RANKL interaction, and was undetectable 30 minutes after the interaction. JNK was phosphorylated 15 minutes after the GST-RANKL

stimulation, however the protein was also rapidly dephosphorylated so that by 30 minutes following GST-RANKL stimulation, phosphorylated forms of JNK were undetectable. The data indicated transient and short-lived activation of ERK, JNK, and p38 in murine osteoclast precursors following the GST-RANKL stimulation.

## EXAMPLE 2

### GST-RANKL RAPIDLY ACTIVATES AKT IN MURINE OSTEOCLAST PRECURSORS

[0203] Osteoclast precursors were isolated, maintained, and manipulated as described in Example 1. Immunoblotting protocol was also the same as in Example 1, except that a primary antibody was specific for phospho-Akt, obtained from Cell Signaling.

[0204] FIGURE 2 shows that there was a detectable phosphorylation of Akt at the time of GST-RANKL stimulation, indicating rapid activation of this protein. Akt is a substrate for PI3 kinase, and in its active state is involved in anti-apoptotic signaling. Akt activation increased with time, i.e. the number of phosphorylated Akt molecules in osteoclast precursors increased with time. Thus, the activation of Akt was greater at 5 minutes than at 0 minutes, and it peaked at 15 minutes following GST-RANKL stimulation.

## EXAMPLE 3

### SYK IS REQUIRED FOR CELL SPREADING AND OSTEOCLAST DIFFERENTIATION

[0205] Osteoclasts are macrophage derived cells and as such are subject to regulation by molecules impacting other members of the immune system. Dap12 is an adaptor protein expressed by NK cells and B and T lymphocytes. Dap12 also mediates maturation of myeloid cells and is expressed by osteoclasts which are dysfunctional in

its absence. We find Dap12<sup>-/-</sup> osteoclast precursors fail to differentiate, in vitro, and the abnormality is partially rescued by high dose M-CSF. The relative paucity of osteoclast number, even in presence of high dose cytokine, is attended by dampened proliferation of precursor cells and their failure to normally migrate towards the osteoclast-recognized matrix protein, osteopontin. Furthermore, Dap12<sup>-/-</sup> osteoclasts generated in high dose M-CSF fail to normally organize their cytoskeleton. The incapacity of Dap12 null cells to undergo normal osteoclast differentiation is not due to blunted stimulation of major RANK ligand (RANKL) or M-CSF induced signaling pathways. On the other hand, when plated on osteopontin, Dap12<sup>-/-</sup> pre-osteoclasts do not activate the tyrosine kinase, Syk, which normally binds to the adaptor protein and transmits downstream signals. Attesting to the importance of the Dap12/Syk complex, Syk deficient macrophages do not undergo normal osteoclastogenesis. Furthermore, the same cells plated onto osteopontin, adhere poorly and fail to phosphorylate c-Src or Pyk2, two kinases central to organization of the osteoclast cytoskeleton.

## INTRODUCTION

[0206] Skeletal remodeling is an ever-occurring process in man which pivots on the activity and recruitment of the unique bone resorbing cell, the osteoclast [Chambers, 2000]. The osteoclast originates by hematopoietic precursors of the monocyte/macrophage family migrating to the bone environment, where, in the presence of the cytokines RANKL and M-CSF, they multinucleate and assume the unique osteoclast phenotype thus acquiring the capacity to degrade mineralized matrix [Boyle et al., 2003; Teitelbaum, 2000].

[0207] RANK signaling, activated by its ligand RANKL which is expressed on stromal cells and osteoblasts [Suda et al., 1999], is mediated by a series of protein kinases including c-Src, c-Jun N terminal kinase (JNK), p38, extracellular signal related kinase (ERK), phosphoinositol-3-kinase (PI-3K), and those activating NF-kB [Darnay et al., 1998; Galibert et al., 1998; Lee et al., 2002; Matsumoto et al., 2000]. M-CSF, which via its receptor, c-Fms, simulates many of the same pathways, promotes proliferation of osteoclast precursors and survival of the mature resorptive

cell [Tanaka et al., 1993; Woo et al., 2002]. Together, therefore, RANKL and M-CSF induce expression of genes, such as those encoding tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor and  $\beta 3$  integrin, which characterize the mature osteoclast and its committed precursors [Faccio et al., 2003b; Kudo et al., 2002]

**[0208]** Once the resorptive cell is in contact with bone, a series of matrix-derived signals, mediated largely through the  $\alpha \beta 3$  integrin, prompt the osteoclast to reorganize its cytoskeleton and assume a unique polarized morphological and functional phenotype [Faccio et al., 2003a; McHugh et al., 2000]. Among the most dramatic of these polarized features is formation of the cell's ruffled membrane which is encompassed by a "sealing zone" or "actin ring" [Teti et al., 1991]. This circular structure, which serves to isolate the osteoclast resorptive microenvironment from the general extracellular space, is characterized by the presence of dense F-actin bundles associated with several cytoskeletal proteins and transmembrane receptors [Akisaka et al., 2001].

**[0209]** The fact that osteoclasts are derived from macrophages, cells which are fundamental to immune recognition, has led to a series of experiments which link the immune system to osteoclast recruitment and function. For example, T-lymphocyte-produced cytokines, including RANKL and  $\text{TNF}\alpha$ , appear central to the enhanced osteoclastogenesis responsible for the bone loss attending menopause and the peri-articular bone erosions of rheumatoid arthritis [Cenci et al., 2000; Romas et al., 2002; Weitzmann et al., 2000]. In this context, the process of antigen presentation, itself, is also a fundamental event in pathological osteoclastogenesis [Jenkins et al., 2002].

**[0210]** These and other insights gained into the means by which osteoclast precursors differentiate and how the mature polykaryon resorbs bone have led to the identification of a number of new anti-osteoporosis therapeutic targets including cathepsin K, c-Src and the  $\alpha \beta 3$  integrin, thus encouraging the exploration of other candidates [Wilder, 2002; Zaidi et al., 2003]. Among the most promising of such

potential targets are intraosteoclastic signaling molecules which also function in the immune system [Kaifu et al., 2003; Paloneva et al., 2002].

[0211] Much of the information in hand regarding the molecular mechanisms of osteoclast formation and function is derivative of studies performed on human disease or genetically manipulated animals, particularly those with osteopetrosis [Marks, 1989; McLean and Olsen, 2001]. Presently, at least 24 genes or loci, the products of which positively or negatively regulate osteoclastogenesis and osteoclast function have been identified [Boyle et al., 2003; Teitelbaum and Ross, 2003]. Some such genes impact formation and/or survival of osteoclast precursors. Others mediate either the ability of these precursors to differentiate or the capacity of the mature osteoclast to resorb bone.

[0212] Dap12 is a transmembrane adapter molecule expressed in a variety of cells of the immune system [Lanier and Bakker, 2000; Tomasello et al., 1998]. In myeloid cells, Dap12 pairs with surface residing receptors including TREMs [Colonna, 2003]. The cytoplasmic domain of Dap12 contains the immunoreceptor tyrosine-based activation ITAM motif, which functions as a docking site for tyrosine kinases, including Syk [McVicar et al., 1998]. Interestingly, deletion of the Dap12 gene, in man, results in Nasu-Hakola disease, which includes skeletal abnormalities in its phenotype [Kaifu et al., 2003; Kondo et al., 2002]. Furthermore, Dap12 is expressed by osteoclasts and, as evidenced by the development of osteopetrosis in mice lacking the protein, is essential for normal osteoclast function [Kaifu et al., 2003; Kondo et al., 2002].

[0213] In this exercise we turned to the molecular pathogenesis of Dap12 deficient osteopetrosis. We find that the failure of Dap12<sup>-/-</sup> myeloid cells to generate osteoclasts can be rescued substantially, but incompletely, by increasing ambient M-CSF. Regardless of cytokine concentration, however, the resorptive capacity and cytoskeleton of Dap12 deficient osteoclasts are deranged. Attesting to the importance of Dap12 associated signaling molecules in osteoclast function, bone marrow

macrophages derived from Syk deficient mice fail to differentiate into mature osteoclasts, and exhibit abnormal cytoskeletal function associated with dampened c-Src and Pyk2 phosphorylation.

## MATERIAL AND METHODS

**[0214] In vitro generation of OCs and bone resorption assay.** Bone marrow macrophages (BMMs), derived from long bones of Dap12<sup>+/+</sup>, Dap12<sup>-/-</sup> [Bakker et al., 2000], Syk<sup>+/+</sup> and Syk<sup>-/-</sup> [Mocsai et al., 2002] mice, were isolated as previously described [Faccio et al., 2003b]. The BMMs were cultured for 3 days in alpha-MEM supplemented with 10%FCS and 1:10 CMG14-12 culture supernatant [Takeshita et al., 2000], which contained the equivalent of 100 ng/ml of recombinant M-CSF. 5x10<sup>4</sup> cells were cultured in  $\alpha$ -MEM containing 10% heat inactivated FBS with 100 ng/ml RANKL [McHugh et al., 2000] and increasing concentrations (from 10- to 100 ng/ml) of mouse recombinant M-CSF (R&D Systems Inc. Minneapolis, Minnesota, USA) in 96-well tissue culture plates. Cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP) activity after 5 days in culture, using a commercial kit (Sigma 387-A, St. Louis, MO). Bone resorption was performed by culturing BMMs exposed to RANKL and M-CSF for 5 days onto dentine. Cells were fixed and stained with FITC-phalloidin to visualize F-actin to detect the actin ring organization or removed by brief treatment with 2N NaOH. Resorption pits were visualized by hematoxylin staining (Sigma).

**[0215] Proliferation Assay.** BMMs from Dap12<sup>+/+</sup> and Dap12<sup>-/-</sup> mice were cultured in the presence of increasing concentrations of M-CSF with or without RANKL (100 ng/ml). After 2 days, viable cell number was calculated using the MTT (3-[4,5 dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; Sigma, St. Louis, MO) method or Cell Proliferation ELISA system (Amersham Pharmacia Biotech, Piscataway, NJ). Briefly, 10  $\mu$ l MTT (5 mg/ml) were added in each well, containing 100  $\mu$ l culture media, and incubated at 37°C for 4 hours. The reaction was terminated with 150  $\mu$ l Isopropanol/0.04N HCl and MTT absorbance determined at the optical density of 570 nm. Six wells were used for each variable and each experiment was

repeated twice. For the ELISA assay, BrdU was added to each well to a final concentration of 10  $\mu$ M. Cells were incubated at 37°C for additional two hours and BrdU incorporation was detected as per manufacturer's instruction.

[0216] Immunoblot. BMMs from Dap12<sup>+/+</sup>, Dap12<sup>-/-</sup>, Syk<sup>+/-</sup>, and Syk<sup>-/-</sup> mice were cultured for 3 days in the presence of 100 ng/ml RANKL and 10 ng/ml or 100ng/ml purified M-CSF, starved for two hours and stimulated for the indicated times with 100ng/ml RANKL or for c-Fms signaling, 10 and 100 ng/ml purified M-CSF. Pre-osteoclasts were lifted with trypsin/EDTA, re-suspended in serum-free medium and plated on 5  $\mu$ g/ml OPN-coated dishes for the indicated times. Cells were washed twice with ice-cold PBS and lysed in the buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2% sodium deoxycolate, 1% NP 40, 1 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 1X protease inhibitor cocktail (Sigma, St. Louis, MO). 40  $\mu$ g of cell lysates were boiled in the presence of SDS sample buffer for 5 min and subjected to electrophoresis on 8% SDS-PAGE. Polyclonal anti-ERK1/2 or anti-phospho-p42/p44 MAPK polyclonal antibodies (Cell Signaling Technology, Inc. Beverly, MA) were used for ERK-MAPK immunoblot. IKB $\alpha$  polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and phospho- IKB $\alpha$ , phospho p38 and p-JNK antibodies were purchased from Cell Signaling Technology. Anti-c-Fos and -Syk were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). phospho-Src antibody was purchased from Cell Signaling and phospho-Pyk2 from Biosource International (Camarillo, CA).

[0217] RNA preparation and reverse-transcription polymerase chain reaction analyses. Total RNA from cultured cells was isolated by the guanidine/phenol method. For reverse-transcription polymerase chain reaction (RT-PCR) analysis, cDNAs were synthesized from 1  $\mu$ g of total RNA using reverse transcriptase and oligo dT primers in a volume of 20  $\mu$ l, and the reaction mixture was finally adjusted to 100  $\mu$ l with TE buffer for PCR analysis. PCR was performed with 1  $\mu$ l of cDNA reaction mixture by using Platinum Pfx polymerase (Invitrogen, Carlsbad, CA) and appropriate primers in a volume of 50  $\mu$ l. The following primers were used: for

cathepsin K, 5'-GGAAGAAGACTCACCAGAAGC-3' and 3'-GCTATATAGCCGCCTCCACAG-5'; for MMP9 5'-CCTGTGTGTTCCCGTTCATCT-3' and 3'-CGCTGGAATGATCTAAGCCCA; for calcitonin receptor 5'-CATTCCTGTTACTTGGTTGGC-3' and 3'-AGCAATCGACAAGGAGTGAC; and for GAPDH 5'-ACTTTGTCAAGCTCATTTCC-3' and 3'-TGCAGCGAACTTTATTGATG-5'.

The samples were transferred to a programmable thermal cycler (Hybaid US, Franklin, MA) that had been preheated to 95°C and incubated for 21 to 40 PCR cycles. Each cycle consisted of a denaturation step at 95°C for 1 minute, an annealing step at 60°C for 1 minute, and an extension step at 72°C for 1 minute. 10 µl aliquots of PCR products were separated by electrophoresis on a 1.5% agarose gel.

[0218] Adhesion and Migration assay. Dap12<sup>+/+</sup> or Dap12<sup>-/-</sup> pre-osteoclasts were lifted with Trypsin-EDTA. Adhesion assay was performed on coverslips coated with 5 µg/ml human osteopontin. 5x10<sup>4</sup> pre-osteoclasts diluted in alpha-MEM+0.5% BSA, with or without 100ng/ml M-CSF, were added to each well. Following 1 hour of incubation at 37°C, cells were washed and TRAP stained. Migration assay was performed using transwell filters, 8 µm pore size (Costar, Cambridge, MA), wherein the lower side of the membrane was coated with the same concentration of osteopontin for two hours at room temperature. In some experiments, 100ng/ml M-CSF was added in the lower compartment of the transwell as chemoattractant. Cells attached to the top surface of the membrane were removed with cotton swabs. Cells that had migrated to the lower side were viewed at 300x magnification, and the number of cells per field determined. Results represent the averages from 15 fields ± SE of a representative experiment.

## RESULTS

### M-CSF partially rescues Dap12<sup>-/-</sup> osteoclasts

[0219] Attesting to failed osteoclast function in vivo, Dap12 deficient mice develop progressive osteopetrosis [Kaifu et al., 2003]. Furthermore, Dap12<sup>-/-</sup> osteoclast

differentiation and function are attenuated in vitro. Because we have shown that a similar osteoclast phenotype, namely that of the  $\beta 3$  integrin-deleted mouse, is rescued by high dose M-CSF [Faccio et al., 2003b], we asked if the cytokine has salutary effects on cells lacking Dap12. When cultured in the presence of RANKL, low dose M-CSF (10ng/ml) induces TRAP expression by Dap12<sup>-/-</sup> bone marrow macrophages (BMMs) and many cells become binucleated (FIGURE 3). In contrast, increasing the concentration of the cytokine to 100ng/ml yields many large, TRAP-expressing mutant polykaryons. On the other hand, although Dap12<sup>-/-</sup> osteoclasts generated in high dose M-CSF approximate their wild type counterpart in size, their morphology is not completely normalized nor are they as numerous.

[0220] To determine if the relative paucity of Dap12<sup>-/-</sup> osteoclasts reflects blunted precursor division, we measured the proliferative rate of early and committed osteoclast progenitors, namely BMMs and mononuclear TRAP expressing pre-osteoclasts, respectively, in the presence of increasing doses of M-CSF (FIGURE 4). Consistent with the failure of Dap12<sup>-/-</sup> cells to generate normal numbers of osteoclasts even in the presence of high dose M-CSF, the proliferative rate of both types of precursors is decreased, particularly as the concentration of the cytokine increases.

Dap12<sup>-/-</sup> osteoclasts fail to normally organize their cytoskeleton or resorb bone

[0221] Since Dap12<sup>-/-</sup> osteoclasts generated in high dose M-CSF differ morphologically from their wild type counterparts we asked if they also differ functionally. To this end, we generated Dap12<sup>-/-</sup> osteoclasts on dentine slices, in high dose M-CSF and stained them with FITC-phalloidin. Documenting that the abnormal shape of these polykaryons is reflective of their cytoskeletal organization, confocal microscopy reveals they are incapable of actin ring formation as the cytoskeletal protein forms numerous clusters distributed peripherally in the mutant cells (FIGURE 5A). In keeping with their dysfunctional cytoskeleton, Dap12<sup>-/-</sup> osteoclasts, established in parallel cultures, are incapable of degrading mineralized matrix as evidenced by a complete absence of dentin resorptive lacunae (FIGURE 5B).

Therefore, high dose M-CSF promotes formation of multinucleated Dap12<sup>-/-</sup> osteoclasts but, unlike its impact on  $\beta 3$  integrin deleted cells [Faccio et al., 2003a], is incapable of normalizing their cytoskeleton.

#### RANK and c-Fms signal normally in Dap12<sup>-/-</sup> cells

[0222] The fact that M-CSF only partially rescues the phenotype of Dap12<sup>-/-</sup> osteoclasts raised the possibility that the cytokine, even in abundance, is incapable of inducing terminal differentiation of the cells. To determine if such is the case, we cultured BMMs, with time, in RANKL and low or high dose M-CSF and measured three markers of osteoclast differentiation by RT-PCR. As seen in FIGURE 6, cathepsin K, matrix metalloproteinase-9 (MMP-9) and calcitonin receptor mRNA levels are diminished in day two and four Dap12<sup>-/-</sup> osteoclastogenic cultures maintained in low M-CSF, but the same markers are completely normalized in high concentration of the cytokine. These data are consistent with the posture that Dap12<sup>-/-</sup> osteoclast precursors fail to fully differentiate in RANKL plus low dose M-CSF because they do not normally transmit intracellular signals induced by these cytokines. To test this hypothesis we first assessed intracellular signaling pathways induced by RANKL and found that three such events, namely activation of the MAP kinase, p38 and AKT, as well as phosphorylation and degradation of I $\kappa$ B $\alpha$ , are indistinguishable from normal in Dap12<sup>-/-</sup> BMMs, indicating that Dap12 is not required for RANK signaling (FIGURE 7A). Furthermore, despite the inability of low concentrations of the cytokine to induce osteoclast formation, Dap12<sup>-/-</sup> pre-osteoclasts normally activate the M-CSF responsive signaling molecules, ERK and c-Fos, regardless of concentration of the cytokine (FIGURE 7B, 7C). Therefore, and again in contrast to  $\beta 3$  integrin deficient osteoclasts [Faccio et al., 2003b], the ability of M-CSF to rescue the Dap12 phenotype is independent on ERK and c-Fos activation.

#### High dose M-CSF is required for spreading and migration

[0223] Cytoskeletal organization of osteoclasts is dependent upon recognition of extracellular matrix. Thus, we assessed the capacity of Dap12<sup>-/-</sup> pre-osteoclasts, in the

presence or absence of M-CSF, to adhere and migrate to osteopontin, an extracellular matrix protein recognized by the osteoclast integrin,  $\alpha v \beta 3$ . Both wild type and Dap12<sup>-/-</sup> pre-osteoclasts attach to the matrix protein within thirty minutes (FIGURE 8A). On the other hand, whereas wild type cells spread on osteopontin within this time frame, those lacking Dap12 do not. Importantly, the spreading defect of the mutant osteoclast precursors is rescued by high dose M-CSF. Similarly, migration to OPN is decreased two fold in cells lacking DAP12 (FIGURE 8B). Adding high dose M-CSF as chemoattractant to the well approximately doubles the migratory capacity of both wild type and Dap12 deficient pre-osteoclasts but fails to rescue the phenotype of the mutant cells. Thus, consistent with the partial rescue of Dap12<sup>-/-</sup> osteoclastogenesis by high dose M-CSF, the cytokine corrects some but not all of the mutant cell's capacity to recognize extracellular matrix.

#### Syk is required for cell spreading and osteoclast differentiation

[0224] The protein tyrosine kinase, Syk, binds to the phosphorylated ITAM domain of Dap12, undergoes its own phosphorylation and mediates Dap12 dependent intracellular events [Lanier and Bakker, 2000]. Thus, to further explore Dap12 downstream signals required for efficient cell spreading and in turn osteoclastogenesis, we analyzed Syk function. Since Syk is phosphorylated in response to integrin engagement [Oberfell et al., 2002] we asked if the tyrosine kinase is activated during cell adhesion to an  $\alpha v \beta 3$  integrin substrate. FIGURE 9A shows that within 30 minutes of plating on osteopontin, wild type pre-osteoclasts phosphorylate Syk whereas their Dap12 deficient counterparts fail to do so. With this information in hand, we turned to the impact of Syk on osteoclast differentiation. We find that RANKL and M-CSF fail to induce Syk phosphorylation in either wild type or Dap12 deficient pre osteoclasts (not shown). On the other hand, Syk<sup>-/-</sup> BMMs generated by transplantation of fetal liver stem cells into lethally irradiated Syk<sup>+/-</sup> mice [Mocsai et al., 2002], fail to differentiate into mature osteoclasts in the presence of the two cytokines regardless of the dose of M-CSF (FIGURE 9B). Attachment of Syk<sup>-/-</sup> pre-osteoclasts to OPN is delayed and, similar to Dap12<sup>-/-</sup> cells, those Syk<sup>-/-</sup> pre-osteoclasts that adhere to the matrix protein fail to spread properly (FIGURE

10A). In keeping with this observation, phosphorylation of c-Src and Pyk2, two signaling molecules which are central to organization of the osteoclast cytoskeleton, is defective in OPN-adherent, Syk deficient cells (FIGURE 10B). Thus, Syk participates in osteoclast differentiation and cytoskeletal organization.

## DISCUSSION

[0225] DAP12 is a membrane-associated protein first identified as an adaptor molecule for a range of activating receptors found on cells of the lymphoid and myeloid lineage [Lanier and Bakker, 2000]. While its small external domain does not appear to bind ligands, two distinctive motifs provide the molecular basis for its activity. The transmembrane region contains a positively charged residue that facilitates non-covalent interactions with receptors. ITAM within the intracellular tail is phosphorylated following interaction between DAP12 and one of its cognate receptors, resulting to recruitment to the site of the src homology-2 (SH2) domains of ZAP-70 or Syk, tyrosine kinases that activate downstream signals.

[0226] Molecules that bind to and hence stimulate signal transduction through DAP12 are primarily on B or T cells. To examine the role of the adaptor in immunity two groups generated mice lacking a functional protein. In one instance the gene was targeted for deletion [Bakker et al., 2000], while the second approach yielded a knock-in mouse in which the ITAM motif was rendered inactive [Tomasello et al., 2000]. In both circumstances the resulting animals are deficient in their innate immune response, a result consistent with the known functions of DAP12 and its activating receptors on lymphoid cells. Subsequent studies have uncovered defects in a number of cellular-based immune functions [Lucas et al., 2002; Sjolín et al., 2002; Wu et al., 2000].

[0227] In addition to its expression by immune cells DAP12 is also found on macrophages [Lanier and Bakker, 2000], where it modulates monocyte differentiation [Aoki et al., 2000]. The activating receptors in these circumstances are incompletely defined, but include myeloid-DAP12 associating lectin-1 (MDL-1), signal regulatory

protein 1 beta (SIRP $\beta$ ) and several members of the triggering receptor expressed on myeloid cells (TREM).

[0228] A potential role of DAP12 and TREMs in skeletal biology was first suggested by Nasu-Hakola disease (NHD). The major manifestations of this rare disorder, found in Japanese and Finnish populations, are a combination of demyelination, leading to early pre-senile dementia and the presence of lipid-filled bone cysts. The Finnish patients have a large deletion in the DAP12 gene while a mutation within the coding region characterizes the Japanese cohort [Paloneva et al., 2000]. In both instances the net result is DAP12 inactivation. Interestingly, a third mouse lacking DAP12 exhibits defects in osteoclast function resulting in osteopetrosis [Kaifu et al., 2003]. More recent studies reveal further heterogeneity in NHD in that a number of affected patients have normal DAP12 expression, but functionally relevant mutations in TREM2 [Cella et al., 2003; Paloneva et al., 2002]. The inability of the cells derived from TREM2 deficient patients to differentiate into osteoclasts suggests TREM2 is upstream of Dap12 in the osteoclastogenic process.

[0229] As part of their studies, Kaifu and co-workers cultured Dap12<sup>-/-</sup> bone marrow cells with M-CSF and RANKL but were unable to generate large numbers of osteoclasts. The few cells expressing TRAP contain only a small number of nuclei and fail to spread. These findings, plus mild osteopetrosis, are similar to the results of similar experiments involving  $\beta$ 3 integrin deficient mice [McHugh et al., 2000]. This observation, plus the fact that much of the phenotype of  $\beta$ 3 null osteoclasts is rescued by increasing the level of M-CSF in the culture medium [Faccio et al., 2003b] prompted us to ask if the same obtains in the case of Dap12. We find this to be so and extended the study to examine selected markers of osteoclast differentiation induced by high dose M-CSF. Confirming our morphological observations, mRNA levels of cathepsin K, MMP-9 and the calcitonin receptor are all enhanced, with time, in Dap12 pre-osteoclasts, by the presence of additional M-CSF throughout the period of osteoclast differentiation.

[0230] It was next considered whether Dap12<sup>-/-</sup> osteoclasts so generated are functional. As part of their capacity to resorb mineralized matrix, osteoclasts form a resorptive organelle at the bone-cell interface, characterized by a ring of cortical actin. Cells lacking DAP12 fail to generate this marker of osteoclast polarization and this defect is not rescued by exposure to an increased concentration of M-CSF. Interestingly, the same high level of M-CSF restores the polarizing capacity of  $\beta 3$ <sup>-/-</sup> osteoclasts but again, in this circumstance, the cells remain incapable of resorption [Faccio et al., 2003b].

[0231] M-CSF, in addition to functioning as an osteoclast differentiation factor, also regulates the proliferation, spreading and migration of both early precursors, in the form of spleen- or bone marrow-derived macrophages, as well as cells committed to the osteoclast lineage by exposure to RANKL [Faccio et al., 2003b; Feng et al., 2002; Stanley et al., 1997]. Because M-CSF does not completely normalize Dap12<sup>-/-</sup> osteoclast number we reasoned the residual defect may reflect dampened precursor proliferation in response to an abundance of the cytokine. In fact, such is the case.

[0232] While increased concentrations of M-CSF markedly increases generation of large osteoclastic cells from Dap12<sup>-/-</sup> progenitors, the mutant polykaryons are incapable of actin ring formation. Thus, while M-CSF has a positive effect on Dap12<sup>-/-</sup> osteoclastogenesis, a profound defect in cytoskeletal organization persists.

[0233] Similar to Dap12, the  $\alpha v\beta 3$  integrin regulates the osteoclast cytoskeleton [Faccio et al., 2003a]. Furthermore, both Dap12 and  $\alpha v\beta 3$  interact with c-Cbl, a key osteoclastogenic signaling molecule, also known to mediate cell spreading [McVicar et al., 1998; Sanjay et al., 2001]. These observations prompted us to ask if like  $\beta 3$ <sup>-/-</sup> pre-osteoclasts, those derived from Dap12 deficient mice, fail to spread on the  $\alpha v\beta 3$  ligand, osteopontin. While wild type cells are fully spread within 30 minutes even in the absence of M-CSF, such does not occur in those lacking Dap12, despite their capacity to attach to the matrix protein. Addition of M-CSF rescues the spreading defect, suggesting that signals emanating from DAP12 enhance the capacity of  $\alpha v\beta 3$

to ligate osteopontin. Haptotaxis and chemotaxis assays, using osteopontin as the coating ligand and M-CSF as the chemoattractant, reveal that once again cells lacking DAP12 are deficient in both parameters and can be rescued by the presence of M-CSF. Since attachment and spreading are both  $\alpha v\beta 3$  dependent and cross talk exists between c-Fms and  $\alpha v\beta 3$  [Faccio et al., 2003b], our findings reveal a complex interaction between the two receptors and Dap12.

[0234] To identify potential effectors of DAP12, we examined the activation of Syk, a tyrosine kinase interacting with and functioning downstream of the adaptor protein in myeloid cells [Lanier and Bakker, 2000]. In our first studies we used wild type and DAP12 null pre-osteoclasts that we allowed to adhere to osteopontin, and then assessed the level of Syk phosphorylation. Only cells expressing DAP12 are capable of activating Syk. To confirm this observation we used pre-osteoclasts generated from macrophages lacking Syk. In this instance we treated the cells with RANKL and both low and high dose M-CSF. Irrespective of M-CSF concentration, absence of Syk precludes formation of fully mature, spread osteoclasts. Furthermore, absence of the enzyme blocks matrix-induced activation of c-Src and Pyk2, two tyrosine kinases essential to  $\alpha v\beta 3$  function in these cells [McVicar et al., 1998; Sanjay et al., 2001]. These morphological findings suggest that, as in other instances in myeloid differentiation, Syk appears to be downstream of DAP12. These studies led us to examine the intracellular signals that follow ligation of RANK and c-Fms, the receptors for RANKL and M-CSF, respectively. Despite the fact that Dap12<sup>-/-</sup> BMMs fail to become mature bone-resorbing osteoclasts, these cells normally activate major signals that follow binding of RANKL to RANK, including those involving NF $\kappa$ B and Akt [Darnay et al., 1998; Galibert et al., 1998; Lee et al., 2002; Matsumoto et al., 2000; Ross, 2000]. Similarly, M-CSF dependent phosphorylation of ERKs and induction of c-Fos, their distal target is intact, an observation that differs from that seen in the  $\beta 3$  null mice, where neither signal is activated by the cytokine [Faccio et al., 2003b; Kudo et al., 2002].

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#### EXAMPLE 4

##### VAV3 IS REQUIRED FOR FUNCTIONAL OSTEOCLASTS

[0283] Osteoporosis is one of the leading causes of morbidity in the elderly and is characterized by a progressive loss of total bone mass and bone density. Bone loss in osteoporosis is due to the persistent excess of osteoclastic bone resorption over osteoblastic bone formation. Osteoclast dysfunction can be dependent on increased cell number and/or enhanced activity, therefore understanding the mechanisms by which the osteoclasts work and/or the factors which regulate their maturation and activity is critical for the cure of the diseases related to bone loss. While factors involved in the differentiative process are in hand, the mechanisms controlling the osteoclast polarization and consequently their activity are still unclear.

[0284] Osteoclasts originate from hemopoietic precursors of the monocyte/macrophage lineage in the presence of M-CSF and receptor activator of NF-kappaB ligand (RANKL). RANK signaling, activated by its ligand RANKL which is expressed on stromal cells and osteoblasts [Suda et al., 1999], is mediated by

a series of protein kinases including c-Src, c-Jun N terminal kinase (JNK), p38, extracellular signal related kinase (ERK), phosphoinositol-3-kinase (PI-3K), and those activating NF- $\kappa$ B [Darnay et al., 1998; Galibert et al., 1998; Lee et al., 2002; Matsumoto et al., 2000]. M-CSF, which via its receptor, c-Fms, stimulates many of the same pathways, promotes proliferation of osteoclast precursors and survival of the mature resorptive cell [Tanaka et al., 1993; Woo et al., 2002]. Together, RANKL and M-CSF induce fusion of the precursors into multinucleated cells, that develop an efficient machinery for dissolving both the inorganic (crystalline hydroxyapatite) and organic (bone matrix rich in collagen fibers) components of bone. With the initiation of bone resorption, the osteoclast undergoes profound morphological changes, polarizes, and at least two distinct membrane domains appear: a ruffled border, the actual resorbing organ which faces the resorbing lacuna; and a sealing zone, the specialized cell-extracellular matrix adhesive structure.

[0285] Attachment of the osteoclast to the proteins of the extracellular matrix is mainly mediated by two adhesive structures: the podosome and the actin ring. Non resorbing osteoclasts, cells which are not yet polarized, are characterized by the presence of highly dynamic adhesive structures, the podosomes, which consist of short bundles of filamentous actin, surrounded by  $\alpha$ V $\beta$ 3 integrin and other cytoskeletal proteins, regularly distributed along the peripheral membrane. Alternatively, resorptive osteoclasts are highly polarized cells that form one or more actin rings, which appear as a continuous band of F-actin around the future resorptive area, and the podosome type dot-like appearance is lost. Since the osteoclasts are highly motile cells, they can detach from one place and attach to the next one, where a new cycle of bone resorption takes place. Therefore, the dynamic changes in the osteoclast cytoskeleton are critical for correct osteoclast activity and, in fact, inhibitors of the actin polymerization can block bone resorption by disrupting the ring of actin and altering the osteoclast morphology.

[0286] Regulation of many actin-dependent processes, such as adhesion, migration, fusion and cell morphology, is under control of the small GTPases, Rho, Rac and

Cdc42. Recent studies have shown that osteoclast activity is in part controlled by members of this family. RhoA is critical for podosome assembly and bone resorption and inhibitors of Rac-GTPases cause dose- and time-dependent cytoskeletal changes, which in turn disrupt the actin ring and decrease the resorptive area. The activation of the small GTPases is tightly regulated by the shift from a GDP-ligated form to a GTP-bound form and is mediated by the Guanine nucleotide Exchange factors (GEFs).

[0287] One of the best characterized GEFs are the Vav Family members, which include Vav1, primarily restricted in its distribution to hematopoietic cells, and the recently discovered Vav2 and Vav3, identified having a more broadly expression. The three members of the Vav Family shares 50-70% identity at the amino acid level. Their Rho-guanosine nucleotide exchange factor function depends on the highly conserved catalytic Dbl-homology (DH) domain. These proteins also share an array of structural motifs characteristic of proteins involved in signal transduction, including pleckstrin-homology, two Src-homology (SH) 3, and a single SH2 domain. Genetic deletion of Vav1 and Vav2 showed their important role in B and T cell development (Tedford K Nat Immun 2001) and T cell antigen receptor-induced pathways. In contrast, deletion of vav3 alone seems not to affect lymphocyte differentiation and signalling, but the combined deletion of all three vav members resulted in a marked reduction in B and T cell number and in a severe failure to activate immunological responses. Although the specific role of Vav3 in vivo has not been establish yet, transient expression of Vav3 in 293T and NIH 3T3 cells demonstrated that ligand stimulation of several receptor tyrosine kinases led to tyrosine phosphorylation of Vav3 and its association with the receptors as well as their downstream signaling molecules, modulating changes in cell morphology (Zeng L, Mol Cell Biol 2000). In platelets Vav3 has been implicated in  $\alpha$ IIb $\beta$ 3 integrin signaling, becomes phosphorylated in response to fibrinogen binding and mediates integrin signaling to the cytoskeleton (Obergfell A, JCB 2002). Moreover, in HeLa cells Vav3 is transiently up-regulated during mitosis, and the enforced expression of Vav3 perturbs cytokinesis and leads to the appearance of multinucleated cells.

[0288] In this study we report an *in vivo* role of Vav3 in regulating the osteoclast function. Deletion of Vav3, alone or in combination with Vav1, leads to a mild osteopetrotic phenotype, due to defective osteoclast function. Vav3 is responsible for correct bone architecture and bone homeostasis, by acting downstream of M-CSF receptor and  $\alpha_V\beta_3$  integrin.

## MATERIALS AND METHODS

[0289] Generation of Vav deficient mice. Germline Vav1<sup>-/-</sup> and Vav2<sup>-/-</sup> mice were described previously (Turner, M. et al. (1997) Immunity 7:451-460; Swat, W. et al. (2003) Int Immunol 15:215-221; Turner, M. and Billadeau D.D. (2002) Nature Reviews 2:476-486). Vav3<sup>-/-</sup> mice were generated as follows: Murine *vav3* genomic clones were obtained by screening a 129 strain phage genomic library (Strategene) with a 438-bp fragment (probe K) corresponding to nucleotides 1140 to 1578 of murine *vav3* cDNA, which was generated as described previously (Moores, S.L. et al. (2000) Mol Cell Biol. 20:6364-6373). This yielded several overlapping *vav3* genomic fragments. One of the clones extending towards the 5' end was used to generate a new probe which included the 5' end of DH domain-encoding sequences (probe H). Using both probes, 4 overlapping clones were characterized that included exons 3 through 17. A targeting construct was made to replace exon 10, encoding part of the DH domain, with the neomycin resistance gene in the reverse transcriptional orientation. A thymidine kinase (tk) gene was used for negative selection of cells with randomly integrated constructs. The targeting construct was linearized and transfected into TC-1 ES cells by electroporation. Positive and negative selection of transfectants was carried out in media containing G418 (0.4 mg/ml) and gancyclovir (1 $\mu$ M). Genomic DNA from individual double-resistant (G418 and gancyclovir) clones was digested with EcoRI and probed with a 5' flanking probe on the Southern blot. Several targeted clones were identified by the presence of a 6.8 kb EcoRI band upon hybridization with the 5' probe, and KpnI digestions were carried out to ensure proper homologous recombination at the 3' end of the targeted region, as identified by hybridization of a 22.0 kb KpnI band with the Neo probe. To rule out any undesired integration events, EcoRI-digested DNA was also hybridized with the Neo probe.

Two independently derived heterozygous Vav3 mutant ES cells were subcloned and injected into C57BL/6 blastocysts to generate somatic chimera mice, which were bred with C57BL/6 females to transmit the targeted allele to the germline. F1 heterozygotes were then intercrossed to generate homozygous mutant mice.

**[0290] Generation of Vav3 antibody.** To generate polyclonal Vav3 antibodies, rabbits were immunized with a fusion protein comprising glutathione S-transferase (GST) and a Vav3 fragment including the DH and PH domains (amino acids (AA) 357-525) expressed in E.coli, using standard methods. The specificity of these antibodies for Vav3 and the lack of cross-reactivity with other family members was confirmed by Western blotting of cell lysates of 293T cells transiently transfected with either an empty vector or expression constructs encoding GFP-fusions of Vav1 or Vav2 or Vav3. Monoclonal antibodies against murine Vav3 were raised by producing a recombinant GST-Vav3 fusion protein (encompassing 230AA including the PH and the N-terminal SH3 domains) expressed in E.coli and purified over a glutathione-Sepharose column (Amersham Pharmacia Biotech), which was used for immunization of Vav3-deficient mice. Splenocytes of the immunized mouse were fused with P3U1 mouse myeloma cells and hybridoma clones were selected and screened for binding to a maltose binding protein-Vav3 fusion protein by ELISA. One positive hybridoma clone, 6D42, IgG1 isotype, was established and used for production of monoclonal antibodies, which showed no cross-reactivity with Vav1 or Vav2.

**[0291] In vitro generation of OCs and bone resorption assay.** Bone marrow macrophages (BMMs), derived from long bones of WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> mice, were isolated as previously described [Faccio et al., 2003b]. The BMMs were cultured for 3 days in alpha-MEM supplemented with 10%FCS and 1:10 CMG14-12 culture supernatant [Takeshita et al., 2000], which contained the equivalent of 100 ng/ml of recombinant M-CSF. 5x10<sup>4</sup> cells were cultured in  $\alpha$ -MEM containing 10% heat inactivated FBS with 100 ng/ml RANKL [McHugh et al., 2000] and increasing concentrations (from 10- to 100 ng/ml) of mouse recombinant M-CSF (R&D Systems Inc. Minneapolis, Minnesota, USA) in 96-well tissue culture plates. Cells were fixed

and stained for tartrate-resistant acid phosphatase (TRAP) activity after 5 days in culture, using a commercial kit (Sigma 387-A, St. Louis, MO). Bone resorption was performed by culturing BMMs exposed to RANKL and M-CSF for 5 days onto dentine. Cells were fixed and stained with FITC-phalloidin to visualize F-actin to detect the actin ring organization or removed by brief treatment with 2N NaOH. Resorption pits were visualized by hematoxylin staining (Sigma).

[0292] Immunoblots. BMMs from WT, Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup>, Syk<sup>+/-</sup>, and Syk<sup>-/-</sup> mice were cultured for 2 days in the presence of 100 ng/ml RANKL and 10 ng/ml purified M-CSF, starved overnight and stimulated for the indicated times with 100ng/ml RANKL for NFκB signaling, and 100 ng/ml purified M-CSF for M-CSF signaling. Pre-osteoclasts were lifted with trypsin/EDTA, re-suspended in serum-free medium and plated on 5 μg/ml OPN-coated dishes for the indicated times. Cells were washed twice with ice-cold PBS and lysed in the buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2% sodium deoxycolate, 1% NP 40, 1 mM NaF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, and 1X protease inhibitor cocktail (Sigma, St. Louis, MO). 40 μg of cell lysates were boiled in the presence of SDS sample buffer for 5 min and subjected to electrophoresis on 8% SDS-PAGE. IκBα polyclonal antibody, p65 antibody, and phospho specific p-ERK antibody were purchased from Cell Signaling Technology, Inc. (Beverly, MA). B-actin antibody was purchased from Sigma (St. Louis, MO). The phospho-tyrosine specific A-G10 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Syk was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

[0293] RNA preparation and reverse-transcription polymerase chain reaction analyses. Total RNA from cultured cells was isolated by the guanidine/phenol method. For RT-PCR analysis, cDNAs were synthesized from 1 μg of total RNA using reverse transcriptase and oligo-dT primers in a volume of 20 μl, and the reaction mixture was finally adjusted to 100 μl with TE buffer for PCR analysis. PCR was performed with 1 μl of cDNA reaction mixture using Platinum Pfx polymerase (Invitrogen Corp., Carlsbad, California, USA) and appropriate primers in a volume of

50  $\mu$ l. The following primers were used: for cathepsin K, 5'-GGAAGAAGACTCACCAGAAGC-3' and 3'-GTCATATAGCCGCCTCCACAG-5'; for MMP-9, 5'-CCTGTGTGTTCCCGTTCATCT-3' and 3'-CGCTGGAATGATCTAAGCCCA; for TRAP, 5'-ACAGCCCCCCTCCACCCCT-3' and 3'-TCAGGGTCTGGGTCTCCTTGG-5'; c-Fos 5'-ATGATGTTCTCGGGTTTCAACG and c-Fos 5'-CAGTCTGCTGCATAGAAGGAACCG; Fra-1 5'-GTGCAAGTGGTTCAGCCCAAGAACTTTT and 5'-GGGTCCTTCTTGTCTCCTTCTGGGATTT for calcitonin receptor, 5'-CATTCCTGTTACTTGGTTGGC-3' and 3'-AGCAATCGACAAGGAGTGAC; and for GAPDH, 5'-ACTTTGTCAAGCTCATTTC-3' and 3'-TGCAGCGAACTTTATTGATG-5'. The samples were transferred to a programmable thermal cycler (Hybaid US, Franklin, Massachusetts, USA) that had been preheated to 95°C, and incubated for 21–40 PCR cycles. Each cycle consisted of a denaturation step at 95°C for 1 minute, an annealing step at 60°C for 1 minute, and an extension step at 72°C for 1 minute. Ten-microliter aliquots of PCR products were separated by electrophoresis on a 1.5% agarose gel.

## RESULTS

### [0294] *Vav3 is the major isoform of Vavs expressed in osteoclasts*

In order to understand the mechanisms regulating the osteoclast activity, we focused on the dramatic cytoskeletal modifications, which occur during the resorptive process. Considering the preponderant role of Vavs Guanosine Exchange Factors in the modulation of the actin cytoskeleton in T and B cells via activation of the Rho Family GTPases, we asked the question whether Vavs could also modulate the osteoclast cytoskeleton. To address this question we first determined which members of the Vav family GEFs were expressed in osteoclasts. We found that osteoclasts express all three Vav members, but interestingly Vav3 is the predominant form, more highly expressed than in other hematopoietic cells (FIGURE 13A). This contrasts to T cells, where Vav1 is the major form and Vav3 demonstrates lowest expression (FIGURE 13B). Consistent with these RNase protection assay results, we found Vav3 protein in BMMs and OCs to be in greatest abundance whereas Vav1 and Vav2 were

expressed at lower levels (data not shown). Also, expression of each of the Vav isoforms remains stable throughout osteoclastogenesis, in contrast to osteoblasts where Vav3 and Vav1 are expressed only in fully differentiated cells (data not shown).

To determine if Vav3 is a major player in regulating osteoclast function, we radiographically and histologically analyzed long bones from 6 week-old Vav3 null mice. Strikingly Vav3<sup>-/-</sup> mice showed enhanced bone density and a visible increase in trabecular bone compared to their wild type littermates on radiographic analysis (FIGURE 14A). Similar to the radiographic analysis, the cortical thickness and the trabecular numbers are significantly increased in mice null for Vav3 (2.7x increase  $p < 0.001$ ). Furthermore, mice lacking both Vav3 and Vav1 showed an even more profound phenotype with an increase in trabecular numbers 3.4 fold ( $p < 0.001$ ) compared to wild type, while the single Vav1<sup>-/-</sup> mice had only a mild increase in bone volume (1.3x  $p < 0.05$ ) (FIGURE 14B and 14C). According to this data, the phenotype of Vav3<sup>-/-</sup> and of Vav1.3<sup>-/-</sup> mice suggests advanced osteosclerosis, while the Vav1<sup>-/-</sup> mice seem to have only a milder defect. Interestingly, in all circumstances, no significant difference in *in vivo* osteoclast number was evident.

We hypothesized that the increase in bone mass of Vav3<sup>ko</sup> and Vav1/3<sup>ko</sup> mice could depend on decreased OC activity and therefore these animals may be protected from bone loss under hormonally-stimulated resorptive challenge conditions. To test this hypothesis, PTH (Parathyroid hormone) fragment 1-34, which induces OC number and bone resorption *in vivo*, was administered for four days by subcutaneous injection every six hours. OC activity was assessed by measuring serum deoxy-pyridinoline (Pyd) levels, an indicator of bone collagen degradation. Notably, while serum Pyd levels are increased 61% in WT mice treated under these conditions with PTH, Vav3<sup>ko</sup> and Vav1/3<sup>ko</sup> mice are protected from the resorptive effect of PTH. To further study whether the bone phenotype of Vav3<sup>ko</sup> mice is intrinsic in the osteoclast lineage, we daily injected RANKL subcutaneously for seven days, treatment known to induce osteoclast differentiation and activation, by binding directly to the RANK receptor expressed in the osteoclast precursors. After one week, the mice were sacrificed and the bone collected for histological analysis. While RANKL treatment increase the

number of osteoclasts in both WT and Vav3<sup>ko</sup>, it induces bone loss, as measured by BV/TV, only in WT animals, but not in Vav3<sup>ko</sup> (data not shown). Of note, the number of osteoclasts in both Vav3<sup>ko</sup> control and RANKL treated mice is 2 times higher than in WT mice, suggesting that lack of Vav3 affects osteoclast function and not their differentiation. In contrast we did not find any difference in the bone mineral apposition rate, measured by tetracycline double labeling (not shown), indicating that the increased bone mass observed in Vav3 deficient mice is not due to upregulated OB activity. In conclusion, these data suggest that Vav3 regulates bone mass *in vivo* possibly affecting the osteoclast phenotype.

**[0295]** *Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts have spreading defect*

In order to analyze if the increased bone mass in Vav3 null mice was dependent on osteoclast function, bone marrow macrophages (BMMs) obtained from all different Vav mutants (Vav1<sup>-/-</sup>, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup>) were cultured for five days in the presence optimal concentration of RANKL and M-CSF to stimulate osteoclast formation. While BMMs from Vav WT and Vav1<sup>-/-</sup> form numerous well spread, TRAP-expressing giant cells, the absence of Vav3 or the Vav1.3 double knock-out completely abrogate formation of large multinucleated cells (FIGURE 15). Cells fuse normally (as demonstrated by the presence of multiple nuclei) but are not capable of extending the peripheral membrane and assuming the typical rounded shape. By immunofluorescence, the actin in Vav3<sup>ko</sup> and Vav1.3<sup>ko</sup> TRAP positive cells clusters in undefined patches randomly distributed in the middle of the cells or at the edge of numerous membrane protrusions, instead of forming the typical row of podosomes along the peripheral membrane (FIGURE 15). These abnormalities are well evident when cells are plated on dentine. In this circumstance, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> cells fail to form visible actin rings and appear more flat (FIGURE 16A). This observation prompted us to measure the osteoclast height, as an index of osteoclast polarization. Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> are respectively 24.5 and 19.2 µm high, versus 39.6 µm for wild type cells, suggesting defects in polarization (FIGURE 16B). Consistent with the morphology data, Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> fail to efficiently excavate bone resorptive pits, confirming the *in vivo* phenotype (FIGURE 16C).

**[0296]** *Normal activation of RANKL and MCSF signaling*

The fact that Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts have abnormal cytoskeleton and do not form an actin ring, raised the possibility that the cells are blocked in early stages of osteoclastogenesis. To determine if such is the case, we analyzed intracellular signaling in response to two osteoclastogenic cytokines, RANKL and M-CSF. NFκB activation in response to RANKL was assessed by analysis of IκBα degradation and by the presence of nuclear p65 (FIGURE 17A and 17B). In all circumstances we did not find any defective NFκB activation. We next analyzed ERK activation, c-Fos and Fra-1 expression in response to M-CSF stimulation, since both prolonged ERK activation and c-Fos are required for efficient osteoclastogenesis and the spreading defect observed in β3 integrin null osteoclasts was correlated with a failure of c-Fos activation. As seen for NFκB activation, normal ERK phosphorylation (FIGURE 18A) an increase in c-Fos after 30 minutes and Fra-1 after 60 minutes of M-CSF stimulation (FIGURE 18B) was detected in all cells, indicating that the differentiation program is intact in Vav1 and Vav3 single and double mutants. Interestingly, despite normal activation of RANKL and MCSF signaling, early osteoclastogenic markers cathepsinK and TRAP were barely detectable in Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> after 2 days in culture, but reached levels comparable to wild type cells within 4 days (FIGURE 19). In contrast, calcitonin receptor, a marker of mature osteoclasts, was defective in Vav3<sup>-/-</sup> and Vav 1.3<sup>-/-</sup> cells even after prolonged days in culture, confirming that these cells, even if they express TRAP, are unable to become fully differentiated osteoclasts (FIGURE 19). Cells lacking only Vav1, however, express all the indicated markers. Therefore, even if not required for commitment to osteoclastogenesis, Vav3 is essential for the final step of osteoclast differentiation.

**[0297]** *Vav3 phenotype is rescued by GFP-Vav3 but not by high dose M-CSF*

We recently found that the spreading defect of osteoclasts lacking the  $\beta 3$  integrin could be rescued by increasing the concentrations of M-CSF in the culture media. Moreover M-CSF is able to rescue the spreading defect of Src deficient osteoclasts. In our system, high dose M-CSF increased only the number of multinucleated cells, but not the spreading of Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts (data not shown).

**[0298]** We then asked the question whether the morphological defect could be rescued by re-expressing the lacking Vav isoform. BMMs were retrovirally transduced with GFP alone or the particular GFP-Vav and Vav expression was analyzed by flow cytometry or by Western blot (FIGURE 20). The positive cells were then cultured in the presence of RANKL and M-CSF and the presence of osteoclasts was evidenced by TRAP staining. GFP-Vav3 completely rescues the defective spreading of Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts (FIGURE 20). In contrast, GFP-Vav1 has only a minimal effect on Vav1.3<sup>-/-</sup> cells. No difference was observed between Vav1<sup>-/-</sup> cells transduced with GFP alone or GFP-Vav1, again indicating that the single deletion of Vav1 does not impact the osteoclast morphology.

**[0299]** *Defective  $\alpha_V \beta_3$  -dependent signaling in Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts*

All the above data suggest that Vav is required for the cytoskeletal reorganization that occurs after the fusion of osteoclast precursors. Since the  $\alpha_V \beta_3$  integrin is a receptor expressed during osteoclast differentiation and involved in the osteoclast interaction with the extracellular matrix, we analyzed whether integrin mediated signals require Vav3 activation. Adhesion of pre-osteoclasts (pre-OCs) to osteopontin (OPN), a substrate recognized by the  $\alpha_V \beta_3$  integrin, induces Vav3 phosphorylation within 30min (FIGURE 21). Complete deletion of Vav3 and Vav1/3 results in substantially diminished Src phosphorylation when cells are plated on OPN, suggesting a novel role for Vavs in regulating the Src/ $\beta 3$  adhesion complex. Considering that Vav phosphorylation was shown to be dependent on the tyrosine kinase Syk, and that Syk<sup>-/-</sup> osteoclasts have spreading defects (data not shown), we asked the question whether Vav3 phosphorylation in response to cell attachment is dependent on Syk. This appears to be the case and in fact Syk<sup>-/-</sup> pre-OCs plated onto OPN for 30min fail to activate Vav3 (FIGURE 21). Having shown that cell adhesion and cell spreading lead to activation of Vav3, we turned

to the possible signal activated by Vav3. Since adhesion to the substratum lead to immediate ERK activation, and Vav has been shown to regulate ERK signaling in T-cells, we analyzed the phosphorylation of ERK in cells adherent versus cells in suspension. Three independent experiments showed that Vav3<sup>-/-</sup> and to a more significant extent Vav1.3<sup>-/-</sup> cells fail to induce ERK activation in response to cell adhesion. As a control, the same cells were stimulated with M-CSF and a strong increase in ERK activation was reached in all mutants (data not shown). These data suggest that Vav3 null osteoclasts have defective integrin signaling. To study in more detail the signal emanated by  $\alpha_v\beta_3$  that requires Vav, we performed immunoblots for p-Src in Vav3 deficient pre-OCs plated onto OPN for 1hour.

[0230] Phosphorylation of the kinase is inhibited in Vav3 null cells, suggesting a novel, essential role for Vav3 in regulating the Src/ $\beta_3$  adhesive complex (FIGURE 22A). Keeping this posture, the activation of Rac in response to M-CSF, an event recently demonstrated as being dependent on  $\alpha_v\beta_3$  integrin activation, is completely inhibited in Vav3<sup>-/-</sup> and Vav1.3<sup>-/-</sup> osteoclasts (FIGURE 22B). Interestingly, the activation of Rho in the same cells occurs normally, indicating a high level of specificity in the Vav substrates (FIGURE 22C).

[0231] Based on this information and on the fact that OCs lacking Syk exhibit defective spreading and phosphorylation of both c-src and Pyk2, we thought that if Syk was playing an important role downstream of  $\alpha_v\beta_3$  integrin through Vav3 activation, mice lacking one allele of Vav3 and one allele of Syk could display a bone phenotype similar to the Vav3 null mice. Histological analysis of Vav3<sup>het</sup>Syk<sup>het</sup> 6 weeks old mice ( $\Delta^{het}$ ) revealed significant increase in bone mass and bone perimeter compared to WT or each single heterozygous animal, to the same extent observed Vav3<sup>ko</sup> mice. Moreover  $\Delta^{het}$  mice, like Vav3<sup>ko</sup> animals, display 2 fold increase in OC number compared to WT, suggesting that the resulting osteoclasts are poorly functional, as further demonstrated by their inability to excavate pits when plated on dentin (data not shown).

[0232] In conclusion we demonstrate Vav3 plays an important role in bone remodeling and disturbing this pathway by partial deletion of Vav3 and its kinase Syk, results in

dysfunctional OCs and increase in bone mass. In summary, the *in vivo* and *in vitro* data suggest a regulatory role for Vav3 in controlling the osteoclast's cytoskeleton and the bone resorption by affecting  $\alpha_v\beta_3$  integrin signalling.

## DISCUSSION

[0233] The Vav protein family comprises three members that play important roles in the proliferation, differentiation and function of B and T cells<sup>9</sup>. The multi-domain structure of all three Vavs<sup>2,3</sup> suggests several mechanisms for their different effector functions. Thus, the Dbp-homology (DH) domain serves as a GEF for several small GTPases, while tyrosine residues or SH2 and SH3 domains act as sites for recruitment of intracellular signaling adaptors that transmit signals to targets as varied as the nucleus and the cytoskeleton.

[0234] Mice lacking Vav-family proteins manifest a number of deficiencies in lymphoid lineages<sup>2,3,9</sup>. Importantly, Vavs participate in the generation and activity of the immune synapse<sup>10,39</sup>, a complex, polarized structure, assembly of which involves re-organization of the actin cytoskeleton (reviewed in<sup>40</sup>). Since OC function also requires actin re-organization and formation of a polarized cell, we reasoned that one or other Vavs may be important in these processes. To test this hypothesis we examined the bone phenotype of mice lacking different Vavs and found that mice lacking Vav3, the major Vav isoform expressed in OCs, have impaired bone resorption and show enhanced bone mass. Thus, quantitative histomorphometry and radiographic examination reveal a significant increase in trabecular bone volume in Vav3<sup>ko</sup> animals, which is further aggravated in mice lacking Vav3 and Vav1, the other Vav protein expressed in OCs. Animals lacking Vav1 also exhibited a significant but much smaller increase in bone mass. The enhanced bone volume measured in Vav3<sup>ko</sup> and Vav1/3<sup>ko</sup> mice did not reflect decreased OC numbers, in fact a two fold increase in the number of TRAP-positive multinucleated cells was evident in untreated Vav3<sup>ko</sup> mice compared to WT. Strikingly, Vav3<sup>ko</sup> and Vav1/3<sup>ko</sup> mice are completely protected from bone loss following PTH administration. In agreement

with these data, also RANKL injection, while promoting a significant increase in the number of Vav3<sup>ko</sup> OCs *in vivo*, which doubles the count of WT cells, fails to induce bone loss, resulting in a further 3.5 fold increase in bone mass compared to WT mice. Vav3<sup>ko</sup> mice are reminiscent of the NIK<sup>ko</sup> and the p62<sup>ko</sup> mice phenotype, being protected by RANKL and PTH induced bone loss. However, while NIK<sup>ko</sup> and the p62<sup>ko</sup> showed only a defect in stimulated osteoclast differentiation, and therefore have normal steady-state bone mass, under this same condition the Vav3<sup>ko</sup> animals have elevated number of OCs and at the same time display over 2 fold increase in bone mass. The fact that the bone mineral apposition rate is similar in WT versus Vav3<sup>ko</sup>, strongly suggests that the phenotype of these animals relays on the osteoclast lineage. Consistent with the capacity of Vav3 null mice to respond to RANKL stimulation with an increase in the number of OCs without losing bone, suggest that Vav3 is regulating the osteoclast activity.

[0235] Having established the *in vivo* phenotype of mice lacking Vav3, we then examined both the capacity of precursors to generate OCs *in vitro* and the resorptive capacity of the resultant cells. Our rationale for these studies was the disparity between *in vivo* and *in vitro* OC formation and function known to exist in other circumstances<sup>34,41</sup>. We first studied intracellular events essential for OC differentiation, namely activation of NFkB and enhanced expression of c-Fos/Fra1 in response to RANKL and M-CSF. The fact that each of the events was normal in Vav3<sup>ko</sup> pre-OCs, indicated that proximal signaling was intact in the absence of the GEF. On the other hand, while OCs lacking Vav3 expressed the markers of OC differentiation, TRAP, MMP9 and cathepsin K, they lacked the calcitonin receptor. Thus this final step of OC differentiation appears to be blocked in cells lacking Vav3.

[0236] Despite the ability of the mutant cells to fuse and form multinucleated TRAP positive cells, Vav3 deficient OCs displayed abnormal morphology and irregular margins. They lacked the capacity to form actin ring, the major structure involved in the formation of the functionally critical sealing zone. As a consequence of cytoskeletal abnormalities, Vav3<sup>ko</sup> OCs failed to excavate bone pits. Thus, Vav3

deficient OCs were not only morphologically and functionally abnormal, but also exhibited defective differentiation, possibly due to the direct or indirect role of Vav3 in gene transcription. The finding of suppressed OC function in the face of elevated polykaryon number *in vivo* is reminiscent of  $\alpha_v\beta_3$ <sup>ko</sup> mice, which are osteosclerotic, but have increased numbers of non-functional OCs that arise as a result of high circulating levels of M-CSF<sup>34,42</sup>. Similarly, while OC number and appearance in DAP12<sup>ko</sup> mice are indistinguishable from those in wild type littermates, the former animals are osteopetrotic<sup>29,43</sup>.

[0237] Of note the dramatic defect of the Vav3<sup>ko</sup> osteoclasts observed *in vitro* resolve in an osteosclerotic and not in an osteopetrotic phenotype *in vivo*, unless the animals are challenged. This apparent controversy is a common feature to several animal models (NIK<sup>ko</sup>, p62<sup>ko</sup>, WASP<sup>ko</sup> and  $\alpha_v\beta_3$ <sup>ko</sup> mice). Two general possibilities present themselves to explain the apparent paradox in the *in vivo* and *in vitro* phenotypes. First, culture conditions may mirror stimulated but not basal osteoclastogenesis. Second, compensatory mechanisms could operate *in vivo*, and therefore many knockout mice exhibit no phenotype until stressed. It is tempting to speculate that Vav3 inhibition may be beneficial for the treatment of some common bone related pathologies, such as rheumatoid arthritis or postmenopausal osteoporosis, which can certainly be considered “stressing condition”, with, in principle, relatively minor toxic effects in bone basal physiology. In this regard Vav3<sup>ko</sup> mice are protected from bone loss in an antigen induced rheumatoid arthritis model, although in this particular model we also observed decreased inflammation, suggesting that Vav3 is playing a role in neutrophil activation (Brugge 2004 JCB).

[0238] Our data suggest that the major role of Vav3 involves re-organization of the OC cytoskeleton, as opposed to differentiation. Multiple factors contribute to regulation of the actin cytoskeleton, among which are members of the Rho family of small GTPases (reviewed in<sup>37,38,44,45</sup>). Impaired activation of Rho and Rac could be responsible for the abnormal morphology observed in Vav3<sup>ko</sup> and Vav1/3<sup>ko</sup> OCs. We tested activation of Rho and Rac in

response to M-CSF and found that activation of Rac, but not Rho, is Vav3 dependent. This observation indicates there is specificity in the activation of these two small GTPases and that it is primarily Rac and not Rho that transduces M-CSF dependent cytoskeletal re-organization in OCs. The importance of small GTPases in regulating actin ring formation in mature OCs has previously been proposed based on studies with C3, dominant negative-Rho or DN-Rac, all of which altered the OC cytoskeleton and the cell's ability to resorb bone <sup>46, 47</sup>. However, the interpretation of these overexpression studies may be problematic as high levels of a dominant negative form of one member of the Rho-GTPase family can inhibit the activation of others. In contrast, our experiments in which OCs lacking Vav3, which specifically inhibited Rac activation, display abnormal morphology and resorptive capacity, provided direct evidence that this GEF is important for OC function.

[0239] In both NK and T cells, Vav1 is activated in response to integrin-mediated adhesion <sup>16,18</sup>. Moreover, Vav1 is phosphorylated in a Syk-dependent manner following adhesion to fibrinogen of CHO cells expressing  $\alpha_{\text{IIb}}\beta_3$ , a receptor for the matrix protein <sup>21</sup>. We recently demonstrated cross-talk between  $\alpha_v\beta_3$  and c-Fms, the receptor for M-CSF and the fact the integrin is required for M-CSF induced cytoskeletal reorganization <sup>34</sup>. Based on these findings we postulated that Vav3 would be phosphorylated in OCs in response to  $\alpha_v\beta_3$  mediated adhesion and find this to be the case. To confirm the role of Vav3 during OC attachment, we analyzed  $\alpha_v\beta_3$  dependent activation of two kinases stimulated by integrin activation, namely c-Src and Erk1/2, in Vav3- and Vav1/3-deficient pre-OCs. Interestingly, both signals were abrogated in the mutant cells. Our findings do not distinguish between direct Vav3 activation of c-Src and Erk1/2 and a situation in which the Vav3 effect is secondary to its capacity to organize the cytoskeleton. Alternatively, Vav3 may regulate  $\alpha_v\beta_3$  conformation a circumstance in which lack of the GEF may maintain the receptor in a low affinity state. The fact, however, that Vav3<sup>ko</sup> OCs adhered strongly to plastic dishes and to OPN coated surfaces (data not shown), but fail to form membrane ruffles and lamellipodia, indicates defects in spreading after binding to matrix. It is therefore possible that the aberrant cytoskeleton of the Vav3<sup>ko</sup> OCs is dependent on

the inability of these cells to adjust to the morphological changes involved in differentiation.

[0240] The abnormalities observed in the Vav3<sup>ko</sup> OCs are reminiscent of the Syk<sup>ko</sup> OCs, which fail to resorb bone, spread upon attachment to OPN, and activate Src downstream of  $\alpha_v\beta_3$  integrin engagement. Moreover the fact that Syk is the kinase involved in Vav3 activation, led us hypothesize that animals lacking one allele of Vav3 and one of Syk could have bone abnormalities. In fact the double het mice showed similar phenotype to the Vav3 null mice, with over 2 fold increase in bone mass under basal condition despite the evated number of OCs. It is possible that complete Syk delition could lead to an osteopetrotic phenotype, but the fact that these animals dye pre-partum, renders impossible the analysis of their bones.

[0241] We propose that Vav3, by activating Rac and  $\alpha_v\beta_3$ -mediated signals, regulates podosome formation and the cytoskeletal changes involved during OC function. Thus, our data suggests that because of the unique function in OC regulation of bone architecture and homeostasis, perturbing Vav3 signaling could represents a novel target for anti-osteoporotic treatments.

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#### EXAMPLE 5

[0289] The previous data in Example 4 demonstrate that ligation of extracellular matrix by the integrin avb3 results in activation of a signal transduction pathway that involves the Syk-Vav3 axis. Furthermore, our findings reveal a key role for Vav3 in osteoclast activation. While it has been reported that the proto-oncogene c-Src links the beta 3 integrin subunit to Vav3, these experiments were performed in part in cell-free systems and, where cellular in nature, involved platelets, cells whose origin is different than osteoclasts and in which the beta 3 subunit does not associate with alpha v (Woodside, DG et al (2002) *J Biol Chem* 277(42):39401-8; Woodside, DG et al (2001) *Curr Biol* 11(22):1799-1804; Arias-Salgado, EG et al (2003) *PNAS USA* 100(23):13928-13302). For these reasons we wished to confirm the beta3/c-Src/Syk linkage in our system. To this end we generated pre-osteoclasts, by treatment of bone marrow macrophages (BMMs) with M-CSF plus RANKL, lifted the cells and either maintained them in suspension or allowed their adherence to the avb3 ligand vitronectin. Cell lysates were prepared, immuno-precipitated with antibodies to the beta3 subunit, followed by western blot analysis. As seen in FIGURE 26A, attachment of cells to vitronectin results in increased interaction between beta3 and both Syk and c-Src. Equal levels of beta3 allow normalization of the data.

[0290] We next repeated the studies described in the previous paragraph, but now reversed the order of immuno-precipitation and western analysis. Thus, pre-

osteoclasts were generated and allowed to adhere to vitronectin or maintained in suspension. Whole cells lysates were generated, immuno-precipitated with anti-Syk and subjected to western blotting using the same Syk reagent (to control for gel loading) and anti c-Src. As can be seen in FIGURE 25B binding of c-Src and Syk requires prior adhesion of cells.

[0291] It is possible that the above findings may arise from adhesion events not mediated by avb3. Thus, to confirm the specificity of our results we isolated BMMs from mice genetically lacking the integrin beta3 subunit and retrovirally transduced these cells with a cDNA coding for full length beta3 protein. Following selection for three days in M-CSF plus puromycin (our retrovirus contains a puromycin selection marker) cells were again treated with M-CSF and RANKL, thus generating pre-osteoclasts. Once again whole cell lysates were prepared and on this occasion immuno-precipitation was carried out using a Syk-specific anti-serum, followed by immuno-blotting with anti-c-Src and, as a loading control, the same Syk antibody. The results in FIGURE 26 show that the presence of beta3 is required for the interaction of Syk and c-Src.

[0292] Syk is a kinase which must be phosphorylated on tyrosine to interact with c-Src. To confirm that integrin ligation indeed results in phosphorylation of Syk pre-osteoclasts, generated as described previously from BMMs with M-CSF and RANKL, were lifted and either maintained in suspension or replated on vitronectin, thus stimulating integrin-based signal transduction. Excess amounts of Syk antibody were used to immuno-precipitate total cellular phosphotyrosine-containing proteins and equal amounts of each lysate (suspended or adherent cells) were analyzed by western blot, using the antibody 4G10, a phospho-specific antibody (FIGURE 27). The data demonstrate that phosphorylation of Syk occurs only upon engagement of vitronectin by avb3.

[0293] Finally, to validate our hypothesis that adhesion of pre-osteoclasts stimulates formation of a complex containing Vav3, Syk and c-Src we once again allowed cells to remain in suspension or to re-adhere to vitronectin. Immuno-precipitation of whole cells lysates with anti-Syk was followed by a series of immuno-blots. FIGURE 28 not only confirms again the interaction between Syk and c-Src, but also demonstrates that the complex contains Vav3.

[0294] In summary, these data reveal a model in which ligation of the integrin avb3 on pre-osteoclasts results in assembly on the heterodimer of a signaling complex containing the kinases Syk and c-Src and the Rac guanosine exchange factor Vav3. Previous studies had established that deletion of Vav3 suppresses the resorptive capacity of osteoclasts and we now demonstrate that activation of this GEF depends on Syk and c-Src. Moreover, absence of either of these tyrosine kinases impairs bone resorption. Taken with our findings that Vav3 is itself critical osteoclast function, the current studies demonstrate a functional linkage between avb3-dependent adhesion and activation of a Syk/c-Src/VAV3 signaling pathway that is central to osteoclast biology.

[0295] This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all aspects illustrate and not restrictive, the scope of the invention being indicated by the appended Claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

[0296] Various references are cited throughout this Specification, each of which is incorporated herein by reference in its entirety.

WHAT IS CLAIMED IS:

1. A method of modulating osteoclast differentiation and/or function in a mammal comprising administering to said mammal an effective amount of a compound or agent that blocks or otherwise inhibits the Syk kinase pathway and/or the Vav3 pathway.
2. The method of Claim 1 wherein said compound or agent is a Syk kinase inhibitor.
3. The method of Claim 2 wherein the Syk kinase inhibitor is selected from the group of 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride, piceatannol (3, 4, 3', 5'-tetrahydroxy-trans-stilbene) and antisense oligonucleotide to Syk kinase.
4. The method of Claim 1 wherein said compound or agent inhibits a protein that phosphorylates Syk or activates a Syk phosphatase.
5. The method of Claim 1 wherein said compound or agent is a Vav3 inhibitor.
6. The method of Claim 5 wherein the Vav3 inhibitor is selected from the group of a Vav family inhibitor, an inhibitor specific for Vav3, antisense oligonucleotide to the Vav family, and antisense oligonucleotide to Vav3.
7. The method of Claim 1 wherein said compound or agent inhibits a protein that phosphorylates Vav3 or activates a Vav3 phosphatase.
8. A method of treating or ameliorating bone disease in mammal comprising administering to said mammal an effective amount of a Syk kinase inhibitor.
9. A method of treating or ameliorating bone disease in mammal comprising administering to said mammal an effective amount of a Vav3 inhibitor.

10. A method of treating or ameliorating bone disease in mammal comprising administering to said mammal an effective amount of one or more of a Syk kinase inhibitor and a Vav3 inhibitor.
11. The method of any of Claims 8-10 wherein said bone disease is selected from the group of osteoporosis, juvenile osteoporosis, osteogenesis imperfecta, hypercalcemia, hyperparathyroidism, osteomalacia, osteomalacia, osteomalacia, osteolytic bone disease, osteonecrosis, Paget's disease of bone, bone loss due to rheumatoid arthritis, inflammatory arthritis, osteomyelitis, corticosteroid treatment, metastatic bone diseases, periodontal bone loss, bone loss due to cancer, age-related loss of bone mass, other forms of osteopenia, as well as in instances where facilitation of bone repair or replacement is desired such as bone fractures, bone defects, plastic surgery, dental and other implantations.
12. The method of Claim 8 or 10 wherein the Syk kinase inhibitor is selected from the group of 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-nicotinamide dihydrochloride, piceatannol (3, 4, 3', 5'-tetradroxy-trans-stilbene) and antisense oligonucleotide to Syk kinase.
13. The method of Claim 9 or 10 wherein the Vav3 inhibitor is selected from the group of a Vav family inhibitor, an inhibitor specific for Vav3, antisense oligonucleotide to the Vav family, and antisense oligonucleotide to Vav3.
14. A method for treating osteoporosis in a mammal comprising administering to said mammal an effective amount of one or more of a Syk kinase inhibitor and a Vav3 inhibitor.
15. The method of Claim 14 wherein the Syk kinase inhibitor is selected from the group of 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]-

nicotinamide dihydrochloride, piceatannol (3, 4, 3', 5'-tetradroxy-trans-stilbene) and antisense oligonucleotide to Syk kinase.

16. The method of Claim 14 wherein the Vav3 inhibitor is selected from the group of a Vav family inhibitor, an inhibitor specific for Vav3, antisense oligonucleotide to the Vav family, and antisense oligonucleotide to Vav3.

17. A method for reducing the risk of non-traumatic bone fracture in a mammal comprising administering to said mammal an effective amount of one or more of a Syk kinase inhibitor and a Vav3 inhibitor.

18. A method for treating bone disease in a mammal comprising administering to said mammal an effective amount of one or more of a Syk kinase inhibitor and a Vav3 inhibitor in combination with one or more other compound for treatment of bone disease.

19. The method of Claim 18 wherein said one or more other compound is an anti-resorptive or anabolic agent and is one or more agent selected from the group of a bisphosphonate, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, a supplemental bone formation agent parathyroid hormone (PTH) or its derivative or fragments thereof, PTH related protein (PTHrp), a bone morphogenetic protein, osteogenin, NaF, PGE<sub>2</sub> agonists, a statin, and a RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomerized form of RANKL.

20. A method of screening for compounds which modulate the differentiation and/or function of osteoclasts comprising selecting compounds which inhibit Syk kinase or the Syk kinase pathway and performing osteoclast assays with said compounds.

21. The method of Claim 20 wherein selecting compounds that modulate Syk kinase or the Syk kinase pathway comprises incubating a Syk kinase with a candidate

compound, and conducting phosphorylation assays wherein a compound's ability to block phosphorylation of Syk or enhance dephosphorylation of Syk is determined.

22. The method of Claim 20 wherein selecting compounds that modulate Syk kinase or the Syk kinase pathway comprises incubating a Syk kinase with a candidate compound, and conducting assays wherein a compound's ability to block the activity of a molecule downstream of Syk or which is modulated or activated by Syk or upon Syk phosphorylation is determined.

23. The method of Claim 22 wherein the molecule downstream of Syk is selected from the group of C $\gamma$ 1 (PLC $\gamma$ ), VAV, CBL, ERK and JNK.

24. A method of screening for compounds which modulate the differentiation and/or function of osteoclasts comprising selecting compounds which inhibit Vav3 or the Vav3 pathway and performing osteoclast assays with said compounds.

25. The method of Claim 24 wherein selecting compounds that modulate Vav3 or the Vav3 pathway comprises incubating a Vav3 with a candidate compound, and conducting phosphorylation assays wherein a compound's ability to block phosphorylation of Vav3 or enhance dephosphorylation of Vav3 is determined.

26. The method of Claim 24 wherein selecting compounds that modulate Vav3 or the Vav3 pathway comprises incubating a Vav3 with a candidate compound, and conducting assays wherein a compound's ability to block the activity of a molecule downstream of Vav3 or which is modulated or activated by Vav3 or upon Vav3 phosphorylation is determined.

27. The method of Claim 26 wherein the molecule downstream of Vav3 is selected from the group of Rho and Rac.

28. A composition for modulating the differentiation and/or function of osteoclasts comprising one or more of a Syk kinase inhibitor and a Vav3 inhibitor.

29. A composition for modulating the differentiation and/or function of osteoclasts comprising a Syk kinase inhibitor.

30. The composition of Claim 28 or 29 wherein said Syk kinase inhibitor inhibits a protein that phosphorylates Syk or activates a Syk phosphatase.

31. A composition for modulating the differentiation and/or function of osteoclasts comprising a Vav3 inhibitor.

32. The composition of Claim 28 or 31 wherein said Vav3 inhibitor inhibits a protein that phosphorylates Vav3 or activates a Vav3 phosphatase.

33. A pharmaceutical composition for treatment or amelioration of bone disease in a mammal comprising a therapeutically effective amount of one or more Syk kinase inhibitor and a pharmaceutically acceptable carrier.

34. A pharmaceutical composition for treatment or amelioration of bone disease in a mammal comprising a therapeutically effective amount of one or more Vav3 inhibitor and a pharmaceutically acceptable carrier.

35. A pharmaceutical composition for treatment or amelioration of bone disease in a mammal comprising a therapeutically effective amount of one or more Syk kinase inhibitor, one or more Vav3 inhibitor and a pharmaceutically acceptable carrier.

36. A pharmaceutical composition for treatment or amelioration of bone disease in a mammal comprising a therapeutically effective amount of a combination of one or

more Syk kinase inhibitor and one or more other compounds for the treatment of bone disease and a pharmaceutically acceptable carrier.

37. A pharmaceutical composition for treatment or amelioration of bone disease in a mammal comprising a therapeutically effective amount of a combination of one or more Vav3 inhibitor and one or more other compounds for the treatment of bone disease and a pharmaceutically acceptable carrier.

38. A pharmaceutical composition for treatment or amelioration of bone disease in a mammal comprising a therapeutically effective amount of a combination of one or more Syk kinase inhibitor, one or more Vav3 inhibitor, and one or more other compounds for the treatment of bone disease and a pharmaceutically acceptable carrier.

39. The pharmaceutical composition of any of Claims 36-38 wherein the one or more other compounds for the treatment of bone disease are selected from the group of a bisphosphonate, a Vav3 or Vav3 pathway inhibitor, a calcitonin, a calcitriol, an estrogen, selective estrogen receptor modulators (SERM's) and a calcium source, a supplemental bone formation agent parathyroid hormone (PTH) or its derivative or fragments thereof, PTH related protein (PTHrp), a bone morphogenetic protein, osteogenin, NaF, PGE<sub>2</sub> agonists, a statin, and a RANK ligand (RANKL), including an osteogenic form of RANKL such as GST-RANKL or other oligomerized form of RANKL.

40. The pharmaceutical composition of any of Claims 33 -38 wherein the bone disease is selected from the group of osteoporosis, juvenile osteoporosis, osteogenesis imperfecta, hypercalcemia, hyperparathyroidism, osteomalacia, osteomalacia, osteomalacia, osteolytic bone disease, osteonecrosis, Paget's disease of bone, bone loss due to rheumatoid arthritis, inflammatory arthritis, osteomyelitis, corticosteroid treatment, metastatic bone diseases, periodontal bone loss, bone loss due to cancer, age-related loss of bone mass, other forms of osteopenia, as well as in instances where facilitation

of bone repair or replacement is desired such as bone fractures, bone defects, plastic surgery, dental and other implantations.

41. An assay system for screening of potential compounds or agents effective to modulate Syk activity of target mammalian cells by interrupting or potentiating the Syk kinase or Syk kinase pathway wherein the test compound or agent is administered to a cellular sample to determine its effect upon the kinase activity or phosphorylation status of Syk, by comparison with a control.

42. An assay system for screening compounds or agents for the ability to modulate the activity of Syk, comprising:

- A. culturing an observable cellular test colony inoculated with a compound or agent;
- B. harvesting a supernatant from said cellular test colony; and
- C. examining said supernatant for the activity of said Syk kinase wherein an increase or a decrease in the activity of said Syk kinase indicates the ability of a drug to modulate the activity of said Syk kinase.

43. A method for detecting the presence or activity of Syk kinase, wherein said Syk kinase is measured by:

- A. contacting a biological sample from a mammal in which the presence or activity of said Syk kinase is suspected with a binding partner of said Syk kinase under conditions that allow binding of said Syk kinase to said binding partner to occur; and
- B. detecting whether binding has occurred between said Syk kinase from said sample and the binding partner;  
wherein the detection of binding indicates the presence or activity of said Syk kinase in said sample.

44. An assay system for screening of potential compounds or agents effective to modulate Vav3 activity of target mammalian cells by interrupting or potentiating the

Vav3 or Vav3 pathway wherein the test compound or agent is administered to a cellular sample to determine its effect upon the kinase activity or phosphorylation status of Vav3, by comparison with a control.

45. An assay system for screening compounds or agents for the ability to modulate the activity of Vav3, comprising:

- A. culturing an observable cellular test colony inoculated with a compound or agent;
- B. harvesting a supernatant from said cellular test colony; and
- C. examining said supernatant for the activity of said Vav3 wherein an increase or a decrease in the activity of said Vav3 indicates the ability of a drug to modulate the activity of said Vav3.

46. A method for detecting the presence or activity of Vav3, wherein said Vav3 is measured by:

- A. contacting a biological sample from a mammal in which the presence or activity of said Vav3 is suspected with a binding partner of said Vav3 under conditions that allow binding of said Vav3 to said binding partner to occur; and
- B. detecting whether binding has occurred between said Vav3 from said sample and the binding partner;  
wherein the detection of binding indicates the presence or activity of said Vav3 in said sample.

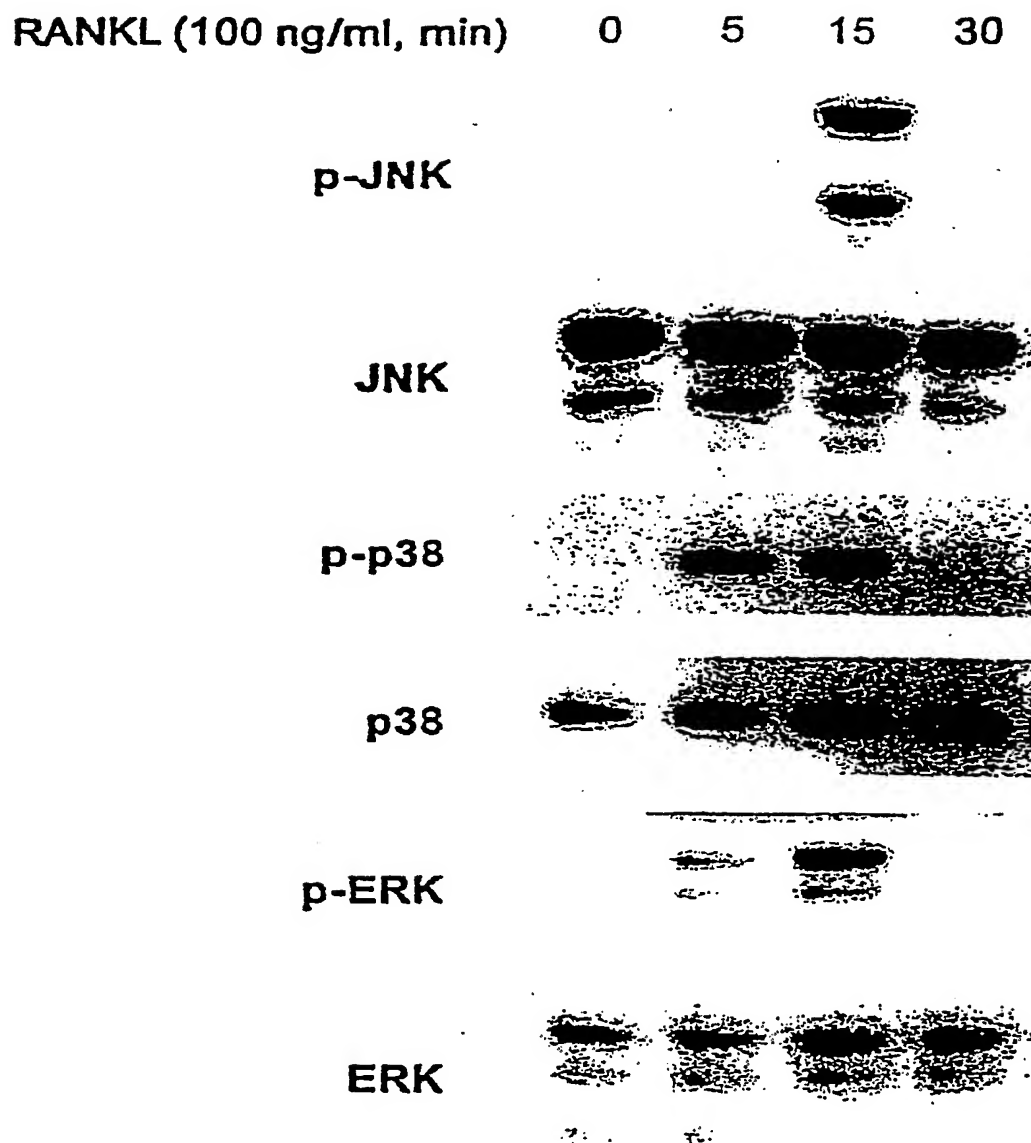


Fig. 1

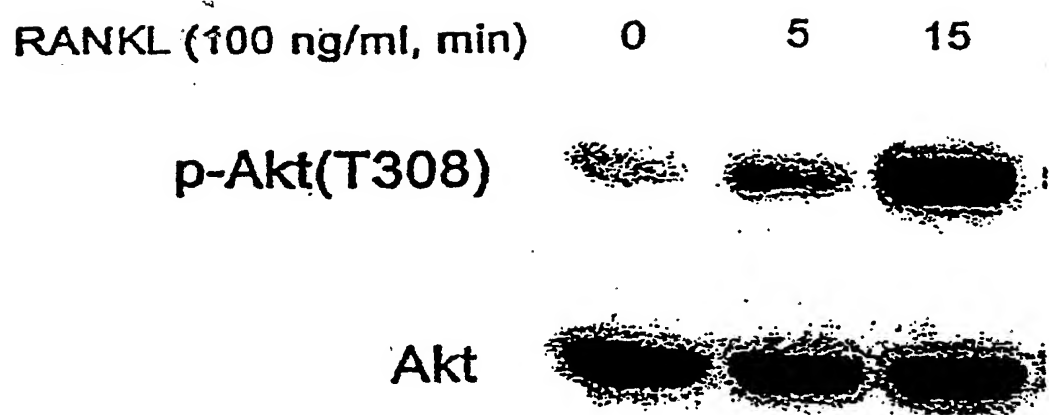


Fig. 2

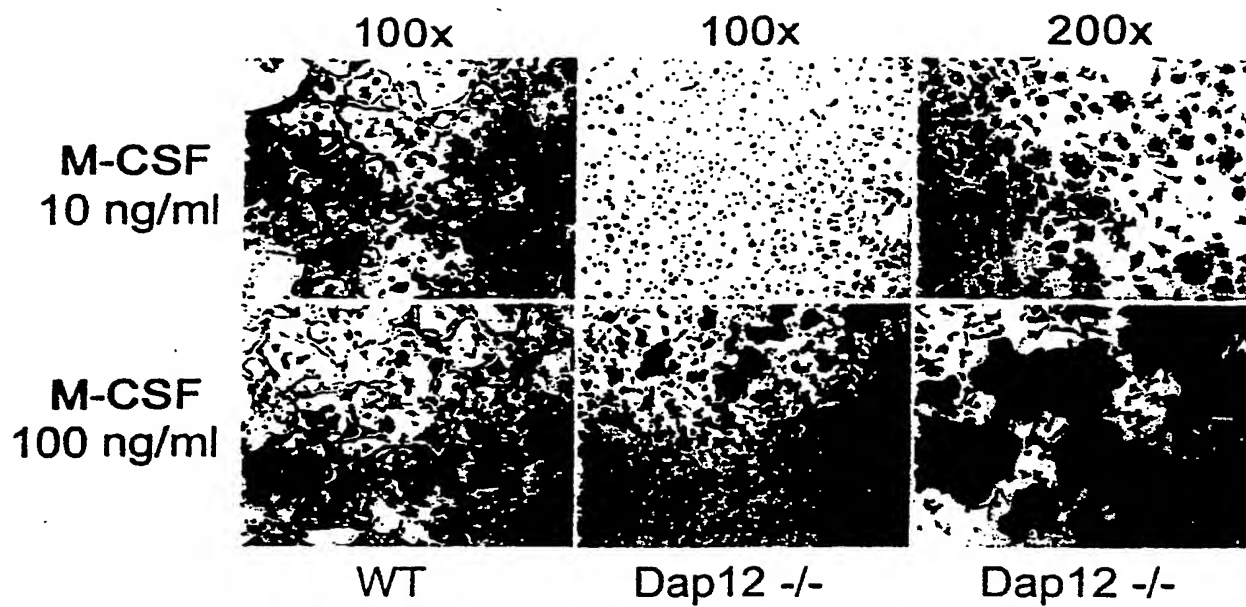


Fig. 3

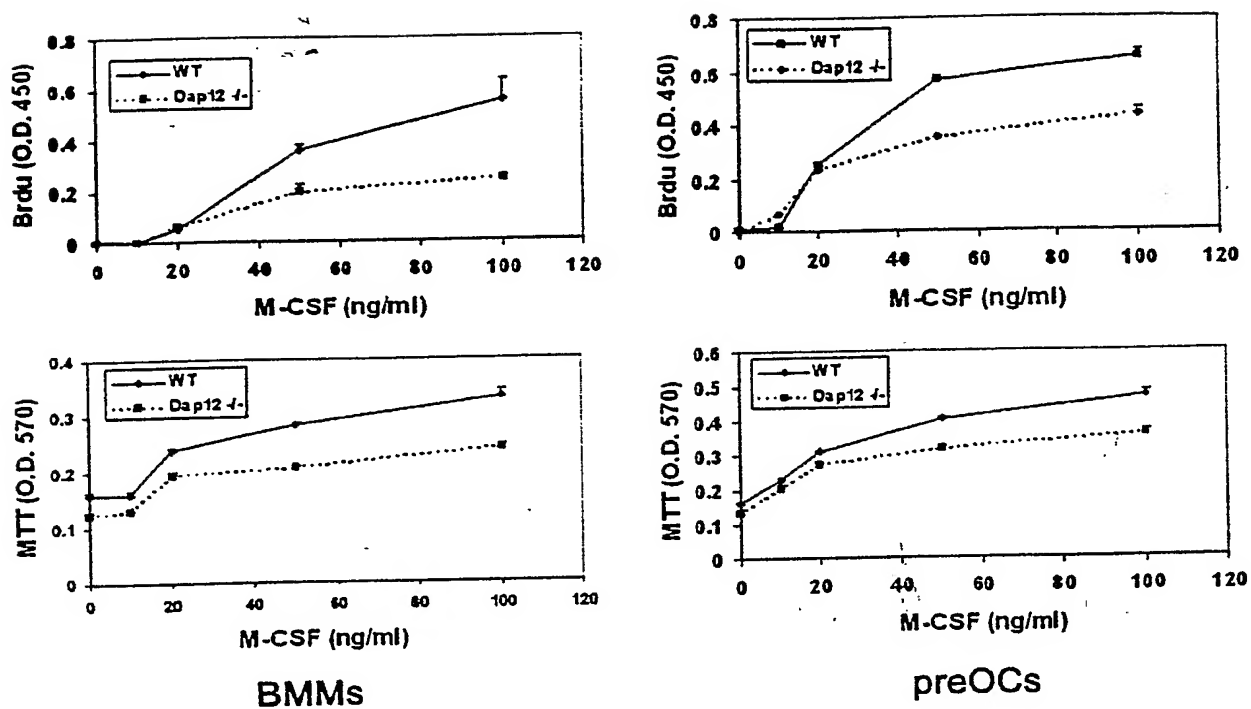


Fig. 4

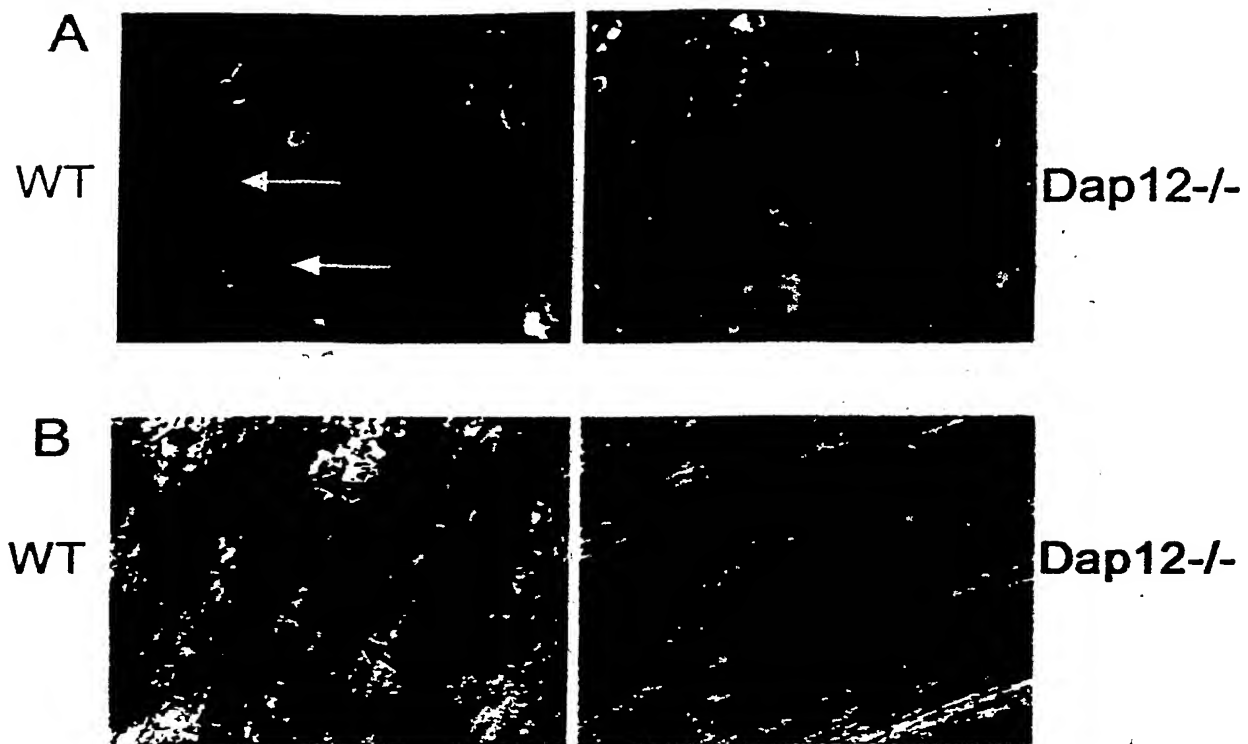


Fig. 5

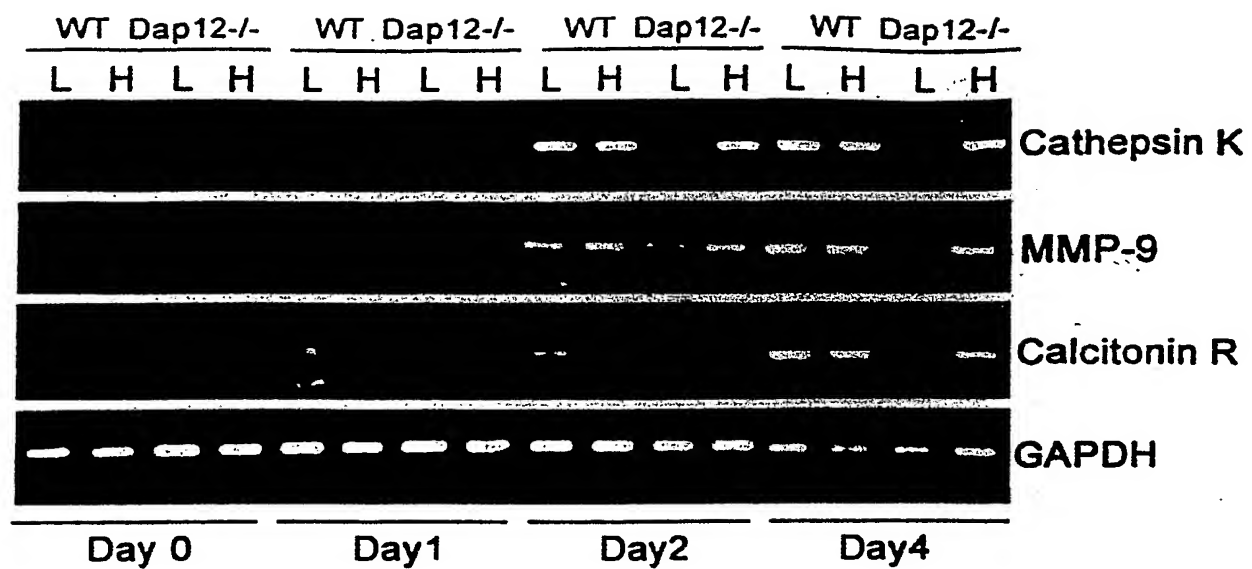


Fig. 6

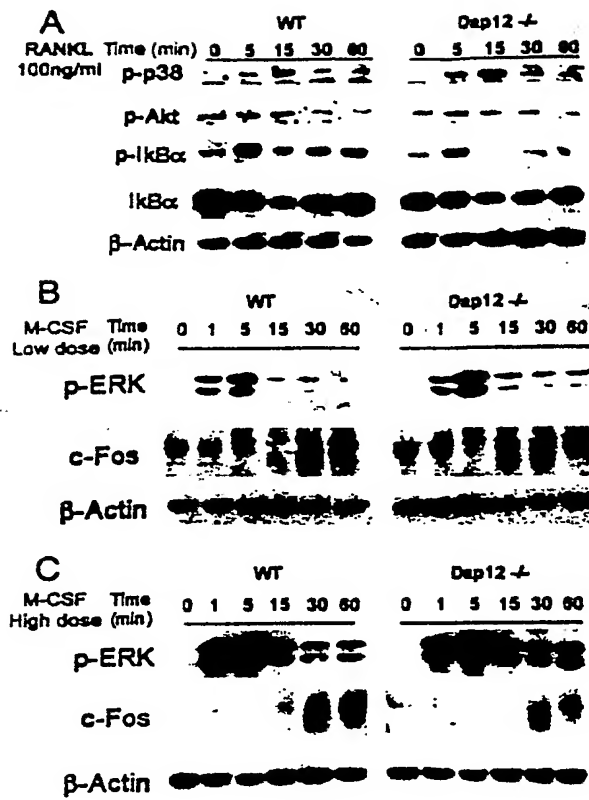


Fig. 7

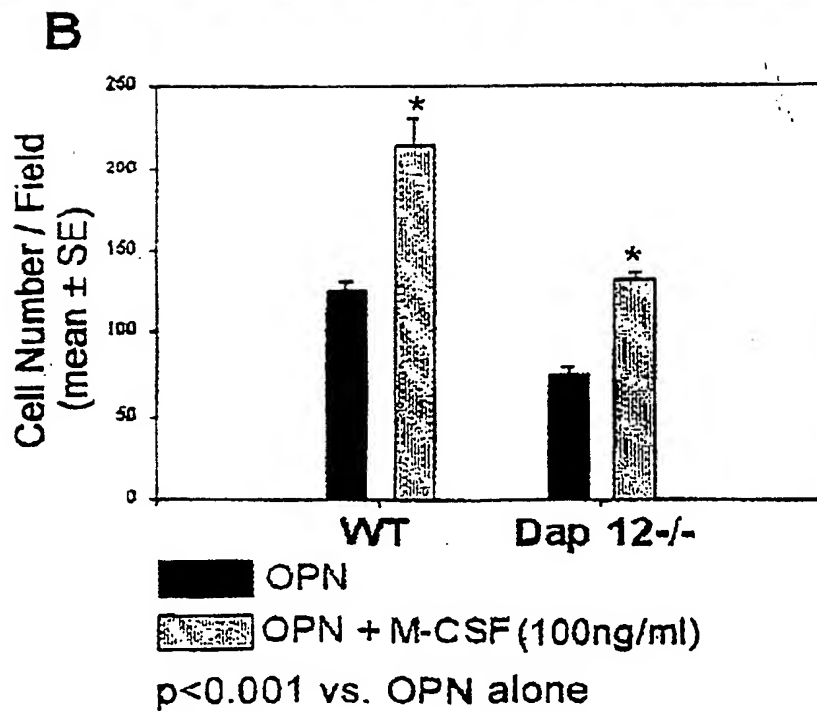
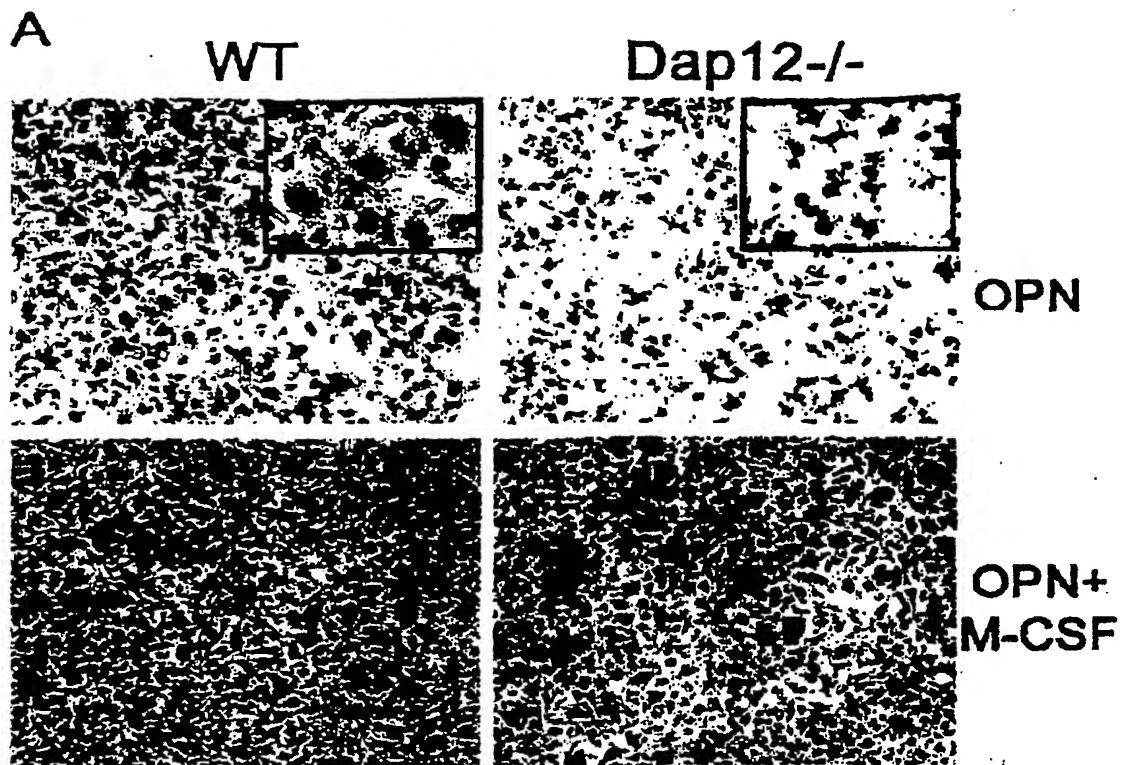


Fig. 8

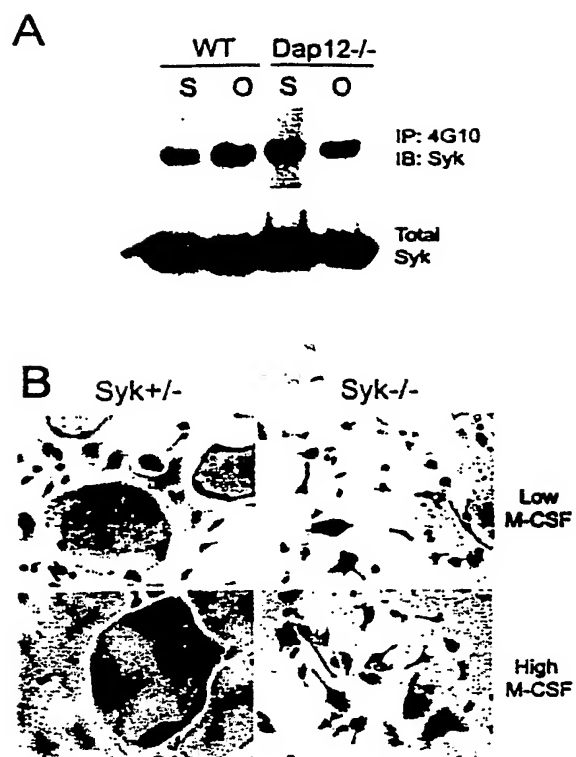


Fig. 9

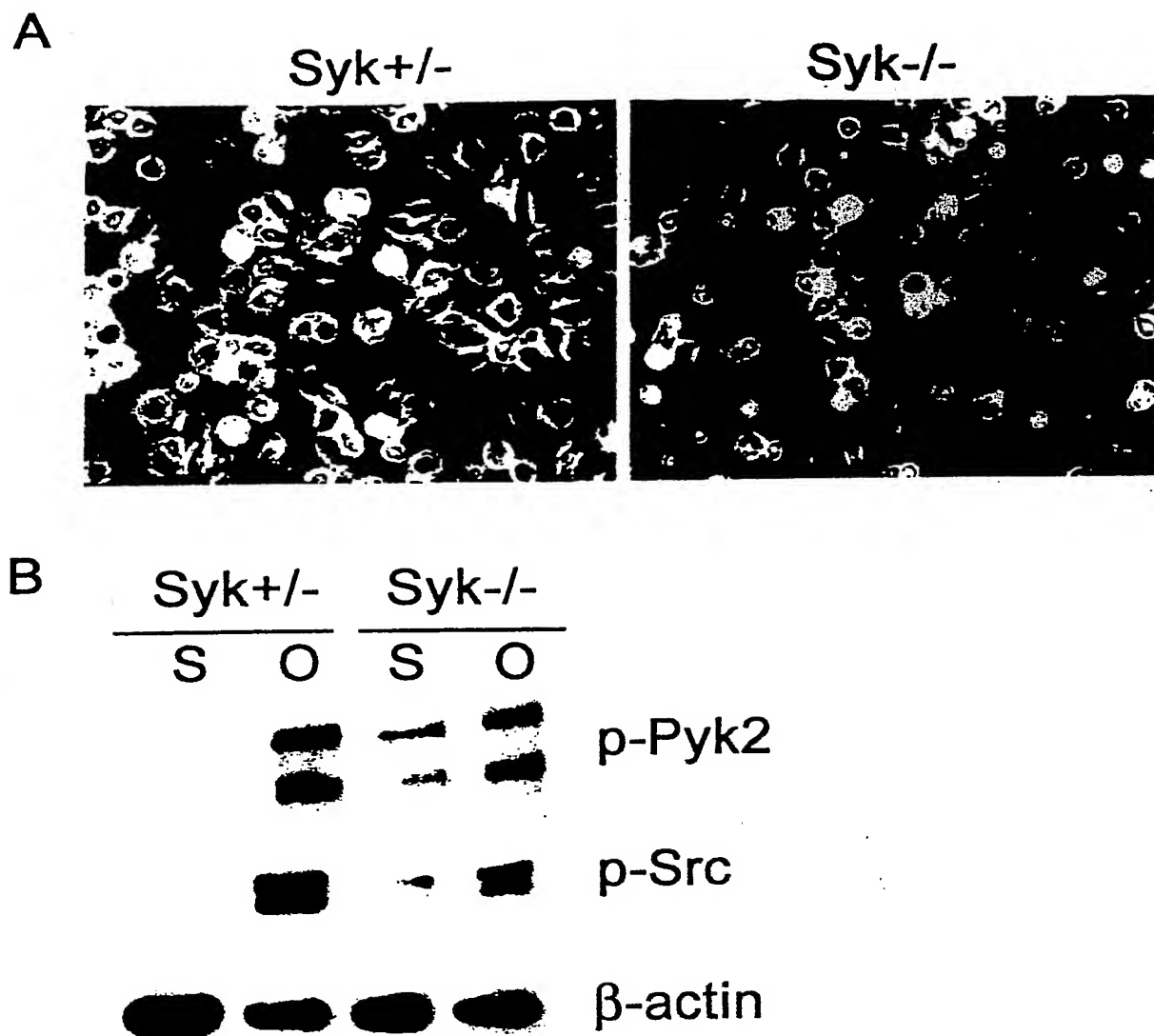


Fig. 10

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181 IVLIGSKTNG KFLIRARDNN GSYALCLLHE GKVLHYRIDK DKTGKLSIPE GKKFDTLWQL
241 VEHYSYKADG LLRVLTVP CQ KIGTQGNVNF GGRPQLPGSH PATWSAGGII SRIKSYSFPR
301 PGRKSSPAQ GNRQESTVSF NPYEPELAPW AADKGPQREA LPMDETVYES PYADPEEIRP
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421 ANVMQQLDNP YIVRMIGICE AESWMLV MEM AELGPLNKYL QQNRHVKDKN IIELVHQVSM
481 GMYLEESNF VHRDLAARNV LLVTQHYAKI SDFGLSKALR ADENYYKAQT HGKWPVKWYA
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601 MYDLMLNCWT YDVENRPGFA AVELRLRNY YD VVN
```

Fig. 11

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481 GGGGTGCAGC CCAAGACTGG GCCCTTTGAG GATTTGAAGG AAAACCTCAT CAGGGAATAT
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841 AAGAAGTTCG ACACGCTCTG GCAGCTAGTC GAGCATTATT CTTATAAAGC AGATGGTTTG
901 TTAAGAGTTC TTA CTGTCCC ATGTCAAAA ATCGGCACAC AGGGAAATGT TAATTTTGGG
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Fig. 12

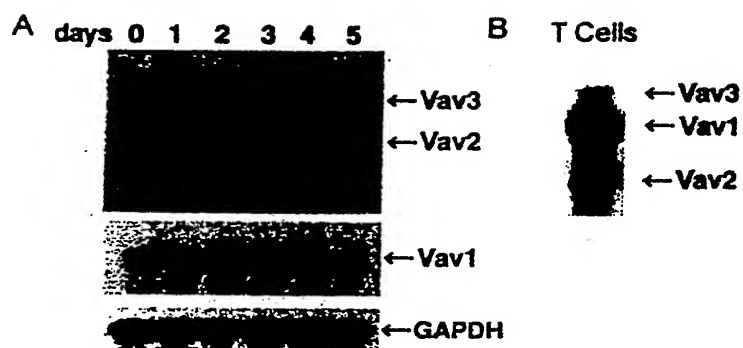


Fig.13

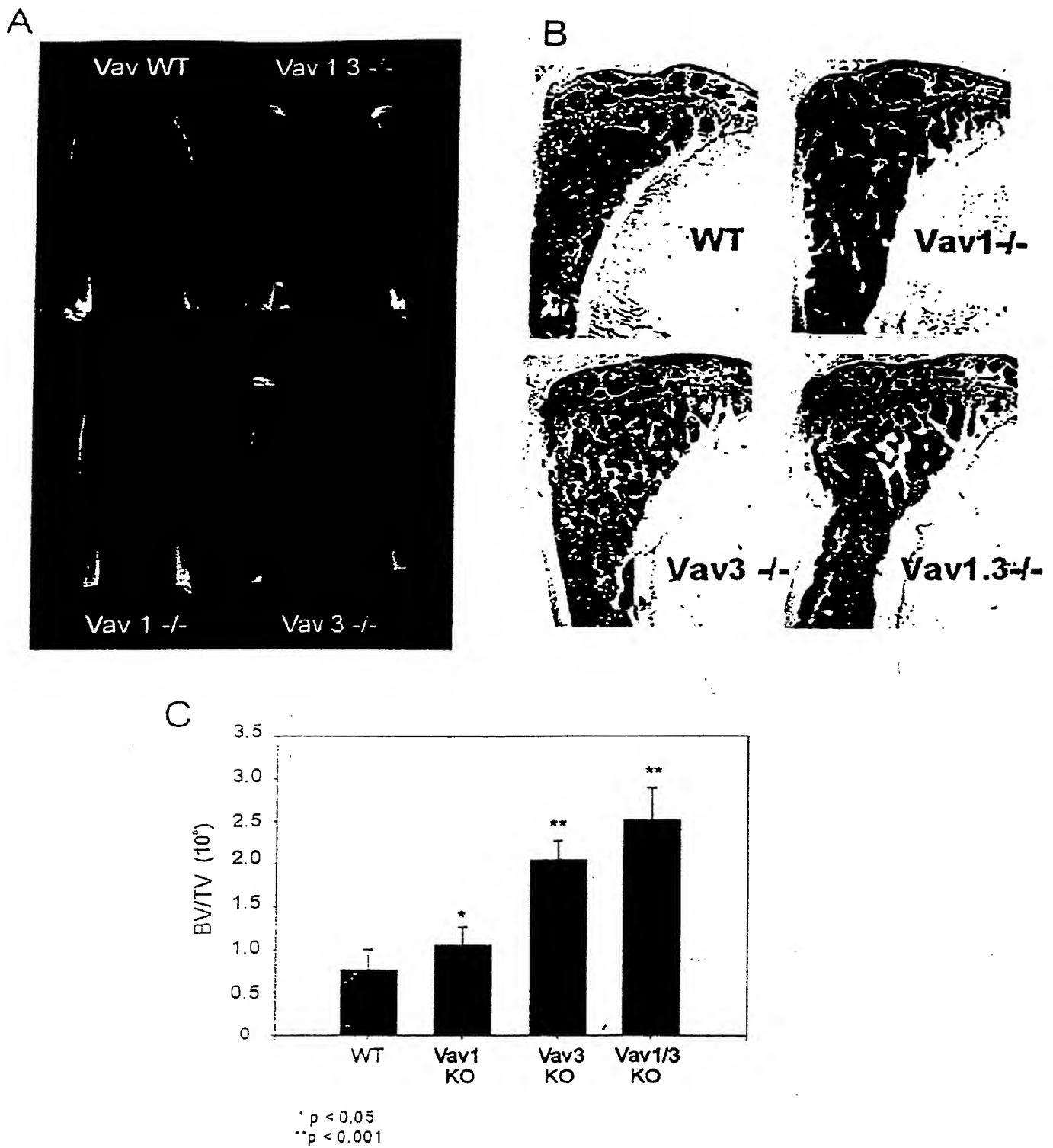


Fig. 14

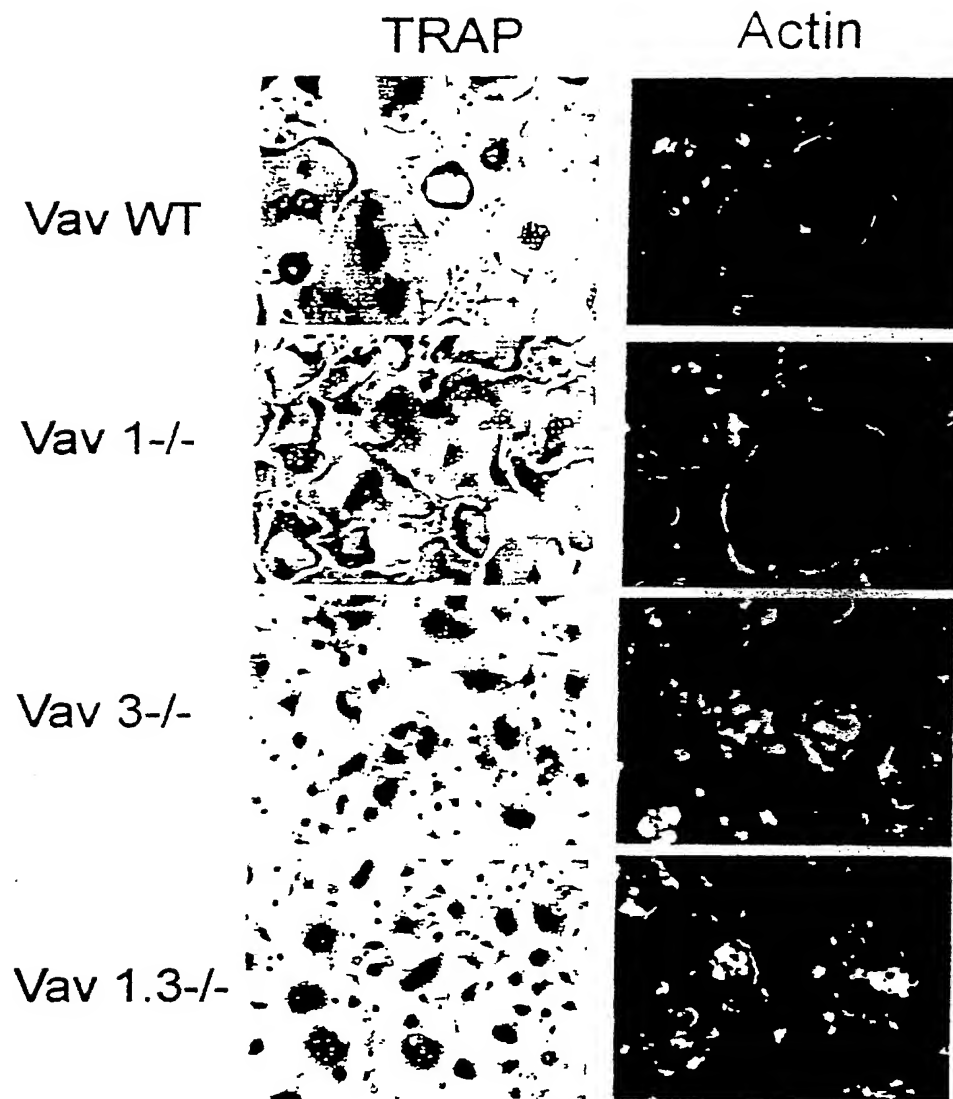


Fig. 15

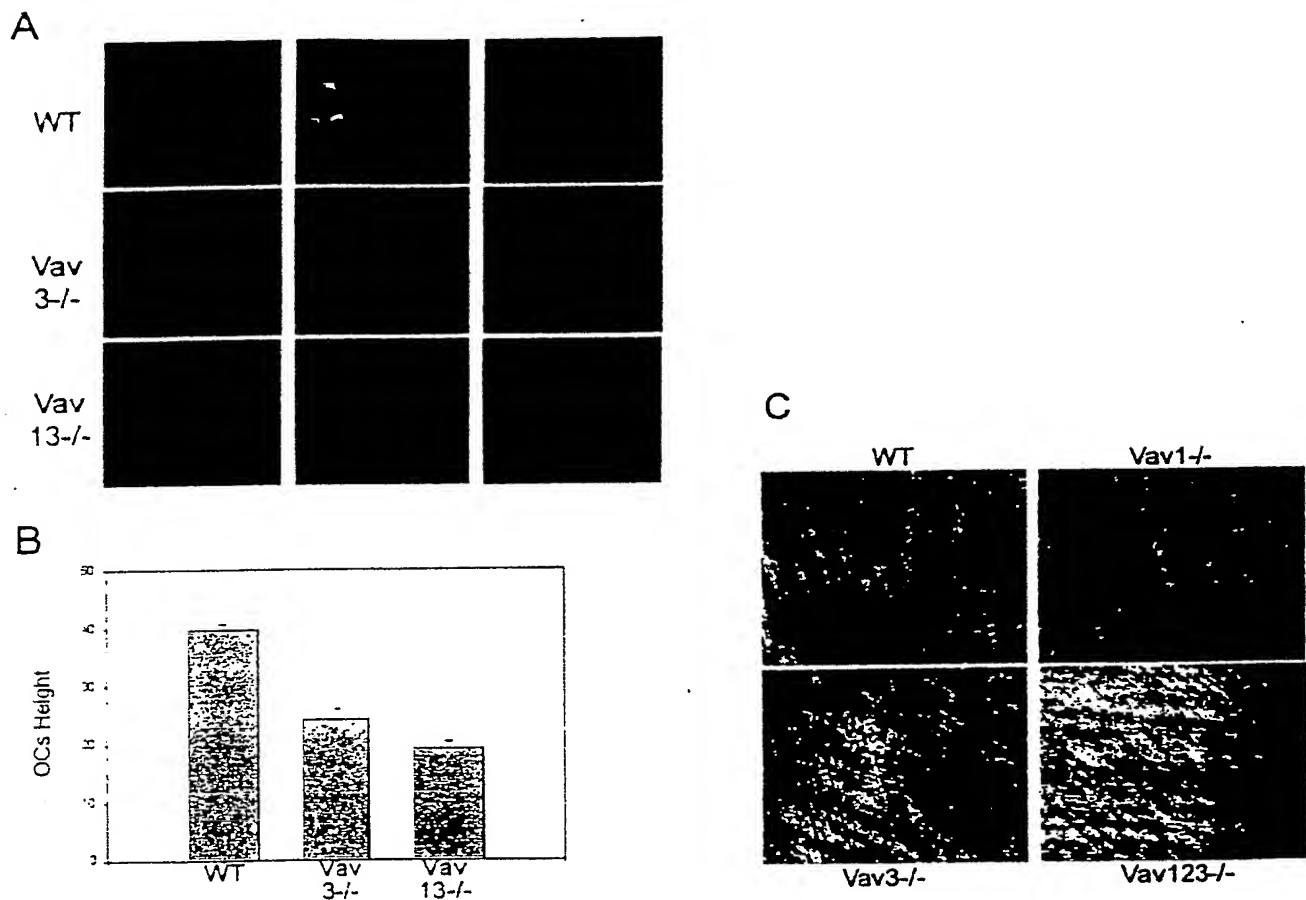


Fig. 16

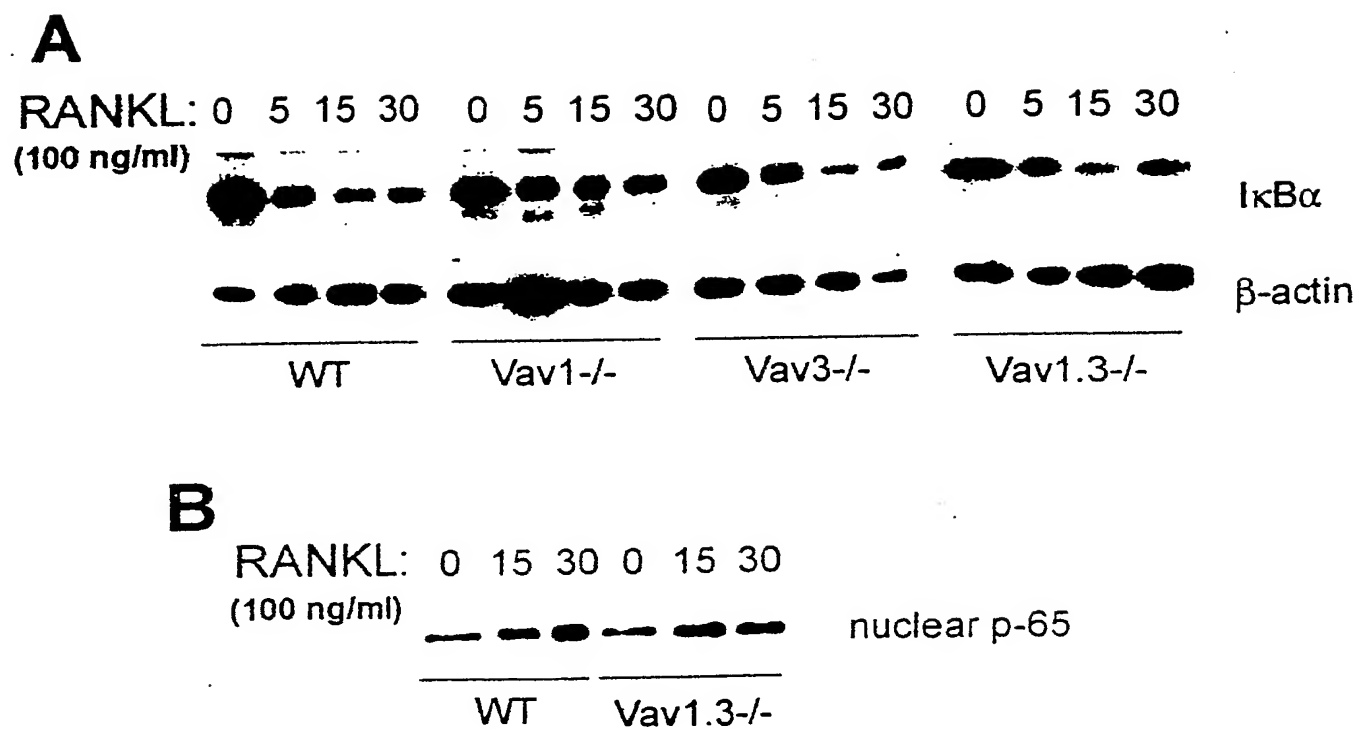
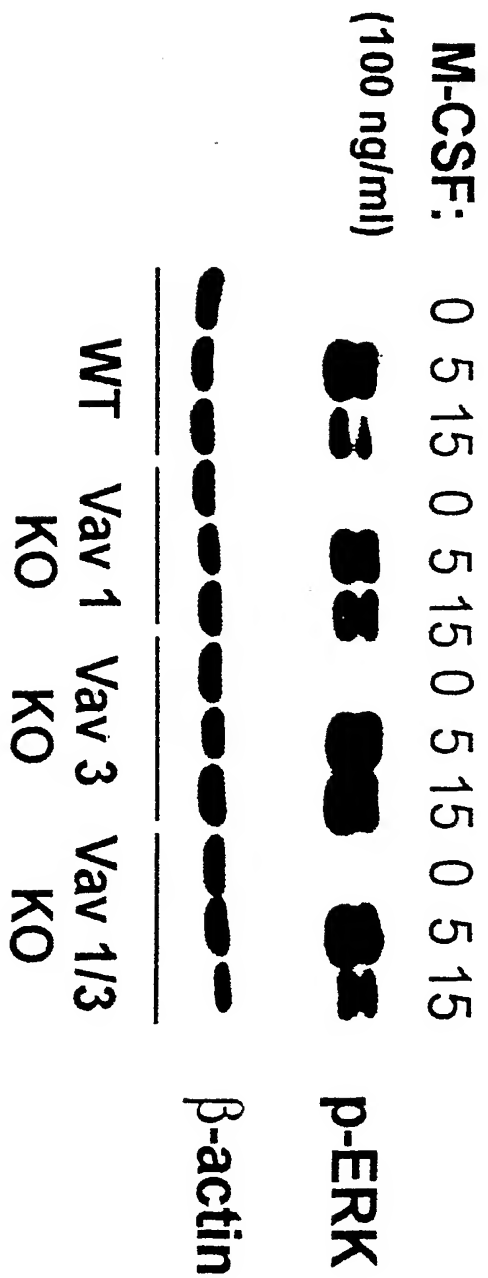


Fig. 17

**A**



**B**

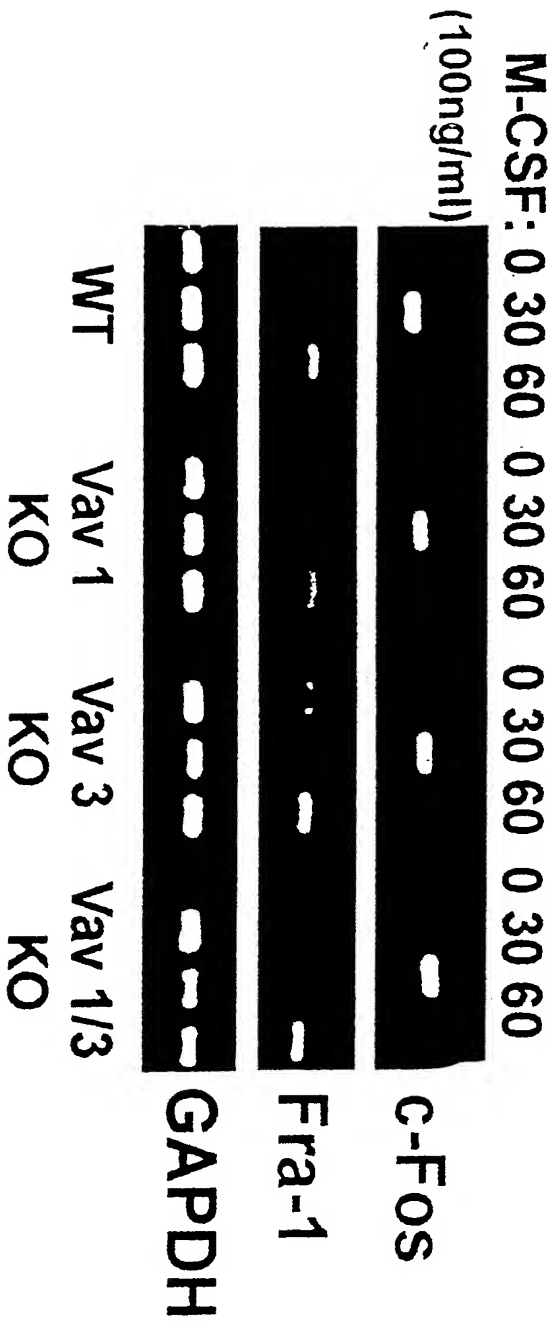


Fig. 18

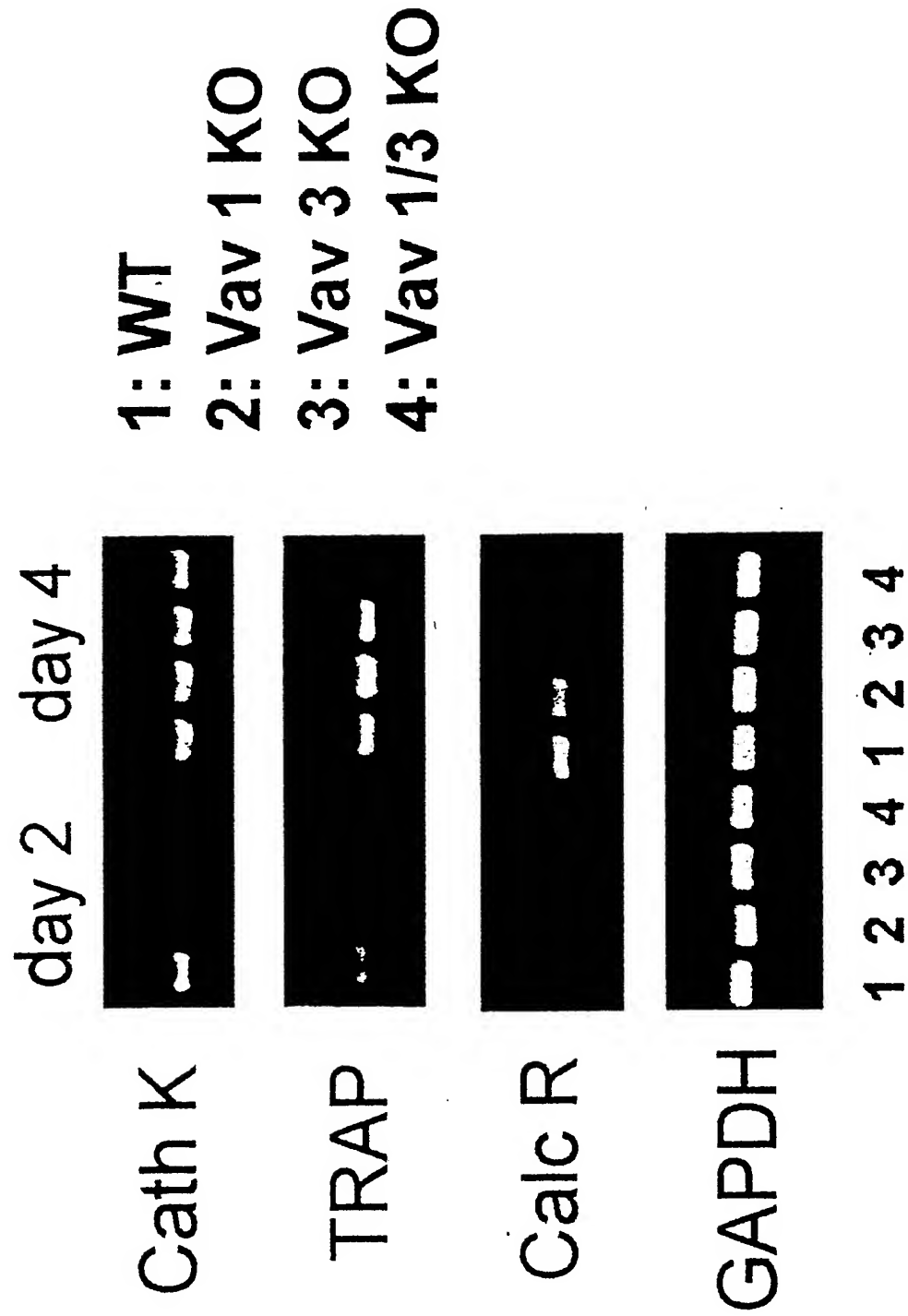


Fig. 19

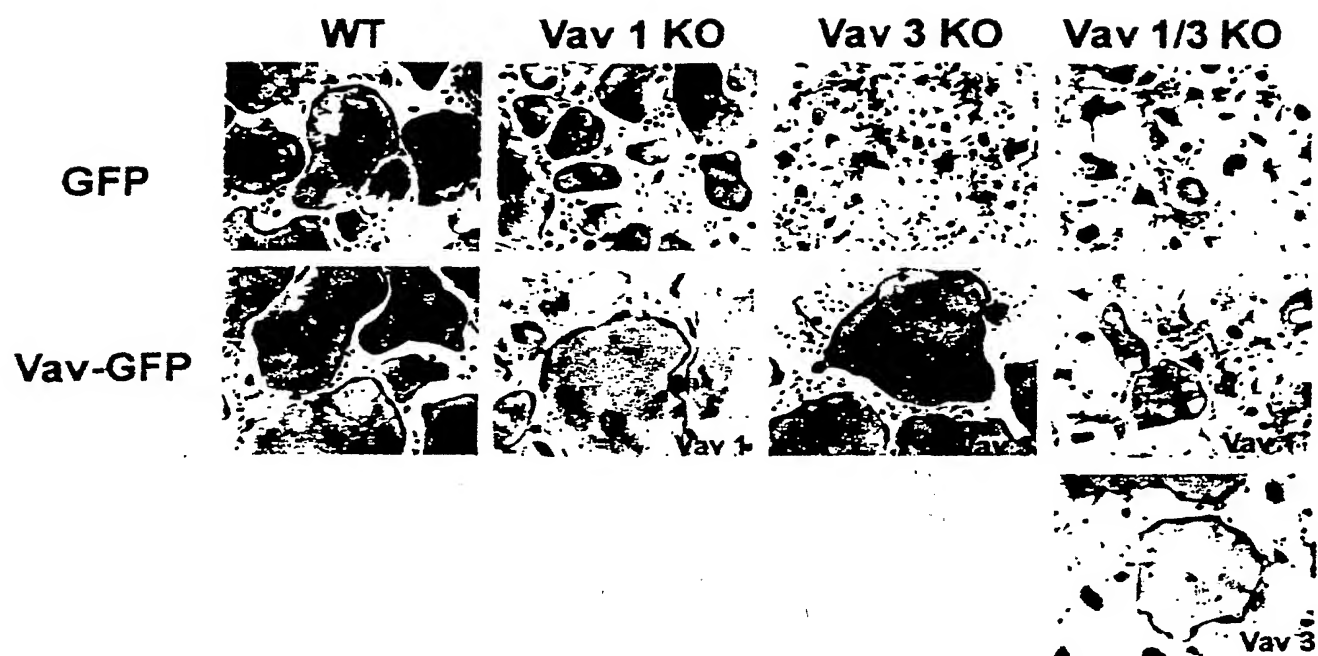


Fig. 20

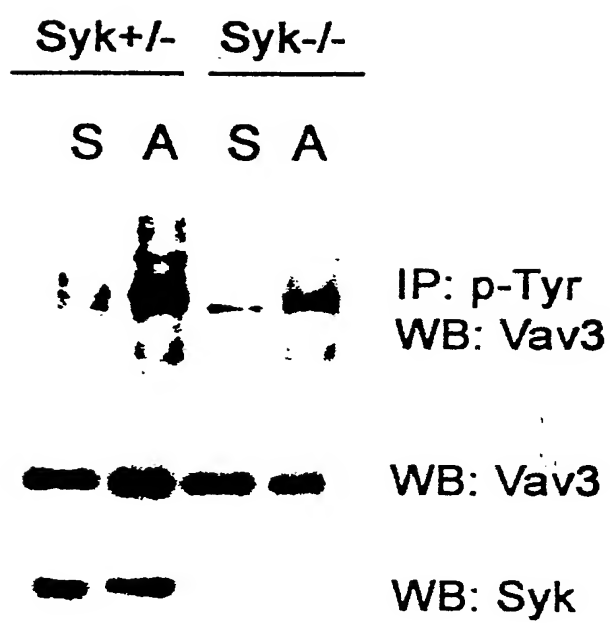


Fig. 21

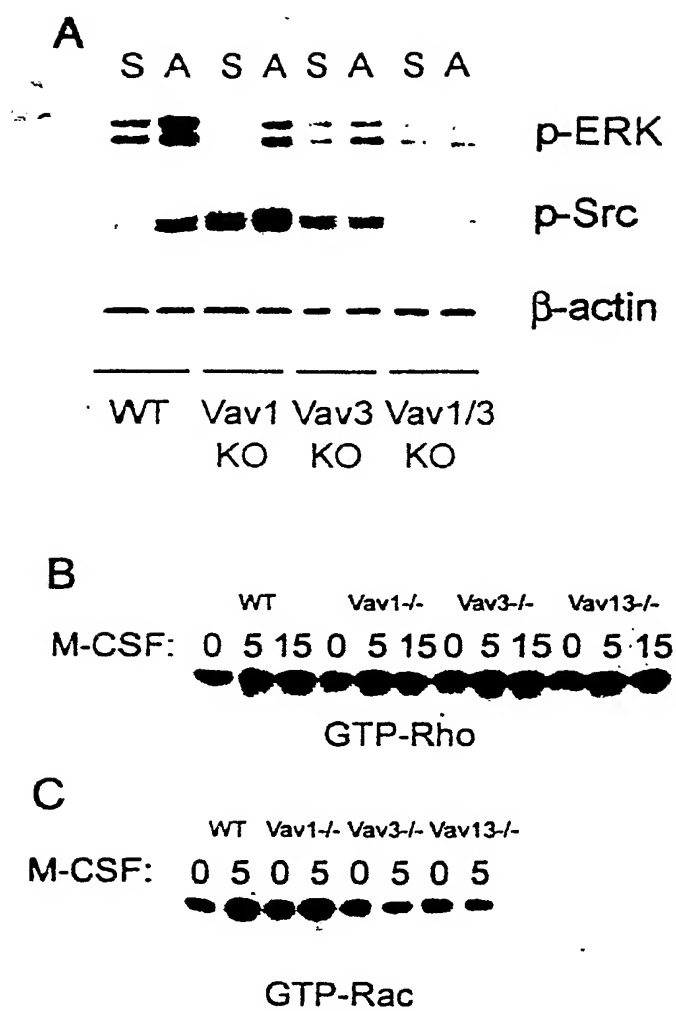


Fig. 22

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661 VPKPVDYSCQ PWYAGAMERL QAETELINRV NSTYLVRHRT KESGEYAISI KYNNEARHIK  
721 ILTRDGGFFH AENRKFKSLM ELVEYYKHHS LKEGFRTLD TLFQFPYKEPE HSAGQQRGNRA  
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Fig. 23

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Fig. 24



Fig. 25A

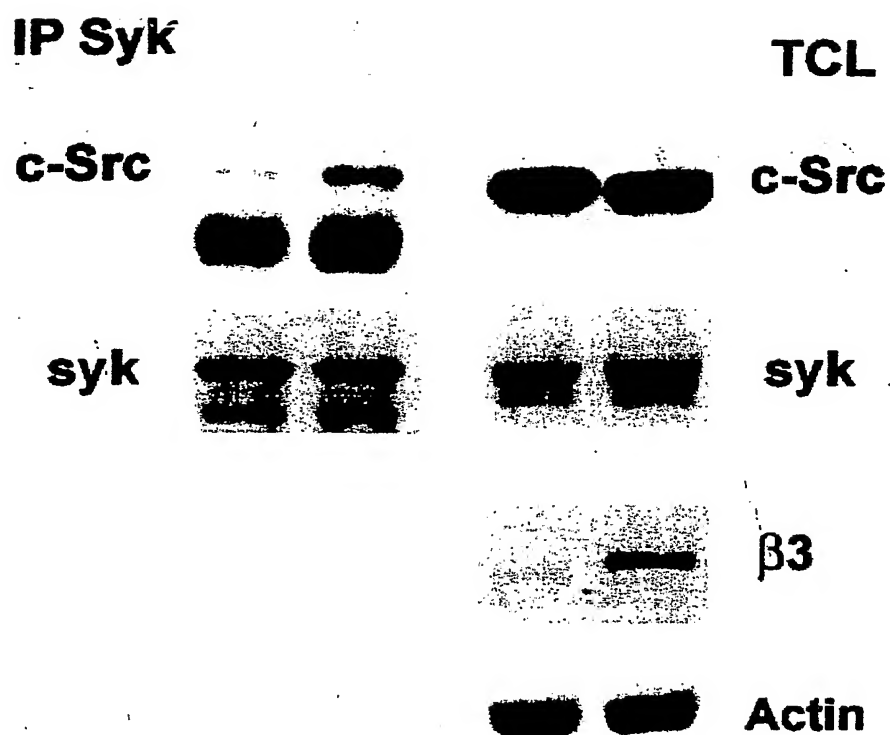


Fig. 25B

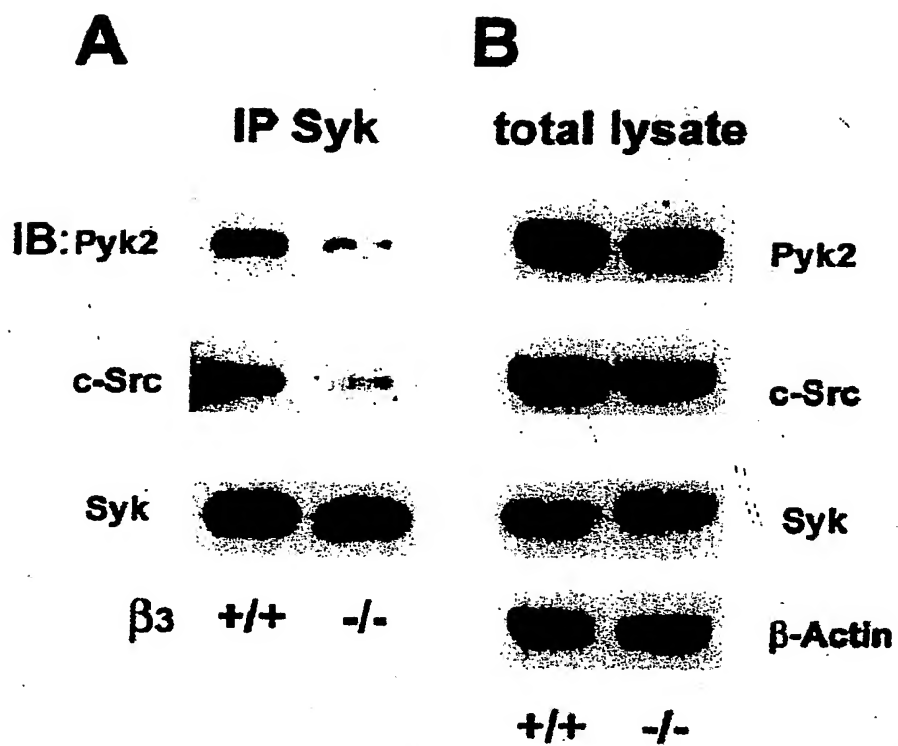


Fig. 26

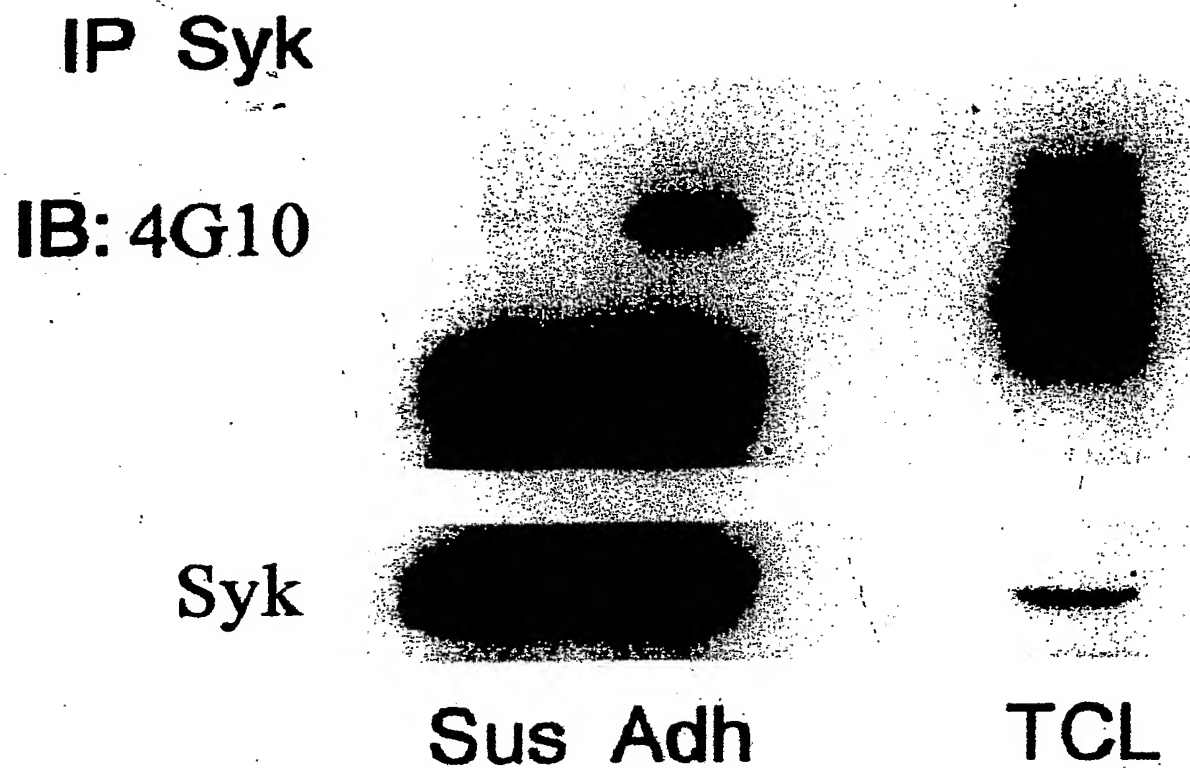


Fig. 27

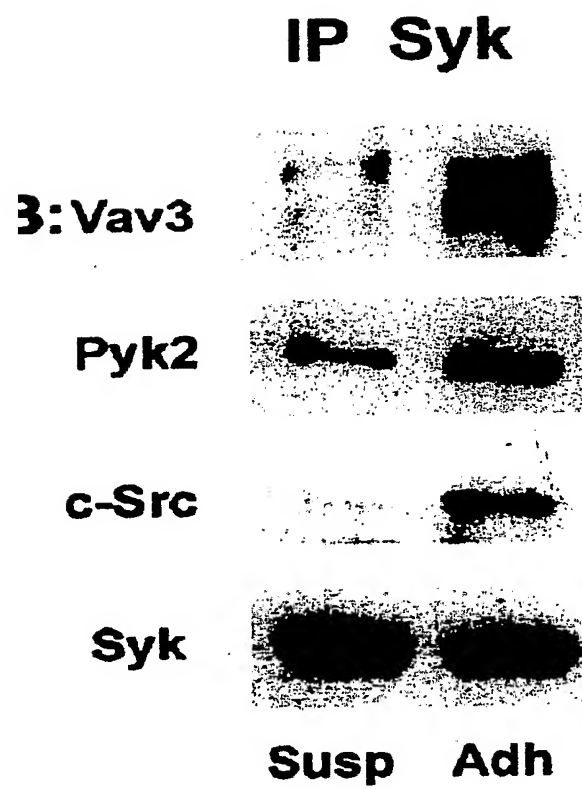


Fig. 28